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Lentiviral target-specific strategy for molecular therapy

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Tese orientada pelo Prof. Doutor João Gonçalves

The opinions expressed in this thesis are from the exclusive responsibility of the author.

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Preface

This thesis presents the research work carried out from March 2008 to July 2011 in the laboratories of Unidade de Retrovírus e Infeções Associadas (URIA) – Centro de Patogénese Molecular (CPM) and Instituto de Medicina Molecular (IMM), under the supervision of Prof. João Gonçalves.

The thesis is divided in four sections. The first one is the introduction, which contains an overview of the gene therapy subjects relevant to this study. The second section describes the materials and methods used throughout this work. In the third one, the results and discussion, it is presented the data obtained during this research work and it is also described and discussed all the trials and attempts necessary to achieve the final results. Finally, in the fourth section it is presented the conclusions and perspectives.

The results shown here are included in a manuscript that is being prepared for submission.

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Abstract

A crucial factor for successful gene therapy is the efficacy of specific gene transfer, which is usually done by lentiviral vectors. Binding specificity and fusion of lentiviral vectors must be provided by envelope glycoprotein domains. The Sindbis virus envelope can pseudotype lentiviral particles and display exogenous protein domains. Previous results from this lab demonstrated that Sindbis envelope can accommodate anti-receptor single-chain antibodies (scFv) and target via cell-specific viral infection. In addition, Dr Irvin Chen laboratory has shown that Protein A-chimeric Sindbis envelope can specifically target cells immunolabelled with anti-receptor IgG via Fc recognition. However, these strategies might present some problems for *in vivo* applications, since there may be non-specific reactions with plasma antibodies and the need for cloning a receptor specific antibody each time a new molecule needs to be targeted. To overcome these problems we developed a new lentiviral vector capable of transducing several cell types in a specific manner without the above constraints, that consists of a chimeric scFv-Sindbis virus envelope that binds fluorescein isothiocyanate (FITC) with high affinity and consequently recognize FITC-conjugated proteins. Therefore, a target cell expressing on its surface a receptor targeted by FITC-conjugated IgG can be infected by this scFv-Sindbis envelope pseudotyped lentiviral vector. Anti-FITC scFv was successfully incorporated at the surface of Sindbis-pseudotyped lentiviruses and could bind to FITC-labelled cells. Using this targeting strategy, we were able, *in vitro*, to target efficiently and specifically Jurkat cells labelled by a CD7 FITC-conjugated antibody. Moreover, we could specifically kill those transduced cells using an HSV-TK/GCV suicide gene strategy. The *in vivo* efficiency of this gene therapy proposal was tested in a mouse model of T-cell acute lymphoblastic leukaemia (T-ALL), which allowed targeting 15.2% of the tumour cells. This provides an alternative strategy to deliver molecular therapeutics using a modular specific targeting with lentiviruses. Moreover, it will overcome the need for new scFv cloning each time a new cell receptor must be targeted and it will avoid the competition by serum antibodies when applied *in vivo,* since the chimeric envelope will only recognize an organic molecule not present in the serum. Although the

strategy herein proposed was applied to a leukaemia model it has the potential to be applied to a broad range of diseases.

Keywords: gene therapy, lentiviral vectors, recombinant antibodies, leukaemia

Resumo

Um factor crucial para realizar uma terapia génica com sucesso é a transferência específica e eficaz de genes, o que normalmente é conseguido através do uso de vectores lentivirais. A especificidade de reconhecimento do alvo e o processo de fusão dos vectores lentivirais deve ser realizado pelas glicoproteínas do invólucro viral. A glicoproteína do invólucro do vírus Sindbis pode pseudotipar lentivírus e apresentar na sua estrutura domínios exógenos de proteínas. Resultados anteriores deste laboratório demonstraram que o invólucro do Sindbis pode acomodar na sua estrutura um fragmento de anticorpo (scFv) que reconhece receptores presentes na superfície celular infectando especificamente esse tipo de células através de um mecanismo alvo-específico. Para além disso, o laboratório do Doutor Irvin Chen mostrou que a glicoproteína quimérica Sindbis-Proteína A pode reconhecer especificamente as células marcadas previamente com IgG, através de um reconhecimento da região Fc da imunoglobulina. No entanto, essas estratégias podem apresentar alguns problemas quando aplicadas *in vivo*, pois pode haver competição inespecífica por parte de anticorpos em circulação, para além da necessidade de se clonar um novo scFV receptor-específico cada vez que se queira fazer o reconhecimento de uma nova molécula. Para ultrapassar estes problemas, desenvolveu-se um novo vector lentiviral capaz de transduzir vários tipos de células de uma maneira específica e sem as restrições acima mencionadas, que consiste na incorporação no invólucro do vírus Sindbis de um scFv que reconhece com elevada afinidade a fluoresceína (FITC) e, consequentemente, é capaz de reconhecer proteínas conjugadas com FITC. Assim, qualquer molécula da superfície de uma célula alvo que seja marcada com um anticorpo conjugado com FITC, será identificada e especificamente infectada por este vector lentiviral. Este anti-FITC scFv foi incorporado com sucesso à superfície de lentivírus pseudotipados com o envelope do Sindbis e foi capaz de reconhecer células marcadas com FITC. Usando esta estratégia *in vitro*, foi possível fazer o direccionamento, de uma forma eficiente e específica, de células Jurkat marcadas por um anticorpo CD7 conjugado com FITC. Além disso, conseguiu matar-se especificamente as células transduzidas usando como estratégia o sistema HSV-TK / GCV. A eficiência *in vivo* desta proposta de terapia genética foi testada num modelo

de ratinho de leucemia linfoblástica aguda das células T, o que permitiu "atingir" 15.2% das células tumorais. Esta proposta de investigação vai fornecer os meios para uma entrega eficiente de bioterapias alvo-específicas utilizando uma estratégia modular de reconhecimento. Além disso, com este sistema deixa de ser necessário realizar novas clonagem de scFv para cada vez que se pretenda fazer o direccionamento para um receptor celular diferente e também evita a competição com anticorpos existentes no soro, quando aplicado *in vivo*, uma vez que o envelope quimérico só irá reconhecer uma molécula orgânica que não está presente no soro. Apesar da estratégia aqui proposta ter sido aplicada a um modelo de leucemia ela tem potencial para ser aplicada a uma vasta gama de doenças.

Palavras-chave: terapia génica, vector lentiviral, anticorpos recombinantes, leucemia

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Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine deaminase
AIDS	Acquired immunodeficiency syndrome
ATCC	American type culture collection
CCD	Charged-coupled device
CGD	Chronic granulomatous disease
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DT-A	Diphtheria toxin-subunit A
EF-2	Elongation factor-2
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ETA	*Pseudomonas aeruginosa*-exotoxin A
FACS	Fluorescence-activated cell sorting
FasL	Factor-related apoptosis ligand
FBP	Folate binding protein
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GCV	Ganciclovir
GFP	Green fluorescent protein
GSI	Gamma secretase inhibitor
GvHD	Graft versus host disease
HA	Hemaglutinin protein (from Influenza virus)
HBV	Human hepatitis B virus
HCL	Hairy cell leukaemia
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidise
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IRES	Internal ribosome entry site
LTR	Long terminal repeat
MLV	Murine leukaemia virus
MOI	Multiplicity of infection
MoMLV	Moloney murine leukaemia virus
NOD	Non obese diabetic
pIX	Adenovirus minor capsid protein
PSA	Prostate specific antigen
RD114	Feline leukaemia virus
RCR	Replication-competent retroviruses
RCL	Replication-competent lentiviruses
RFP	Red fluorescent protein
scFv	Single-chain variable fragment

SCID	Severe combined immunodeficiency
SIN	Self-inactivating
siRNA	small interfering RNA
SIV	Simian immunodeficiency virus
T-ALL	T-cell acute lymphoblastic leukaemia
TCR	T cell receptor
TK	Thymidine kinase gene from HSV
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TU	Transducing units
VSV-G	Vesicular stomatitis virus-glycoprotein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Objectives

Gene therapy has been developed for the treatment of various inherited disorders, as well as acquired diseases, such as cancer and human immunodeficiency virus (HIV) infection. An essential tool for this gene therapy is the delivery vector, which can be viral or non-viral. The remarkable advances in the development of delivery vectors and the knowledge of the molecular mechanisms of disease have contributed for the progress of human gene therapy. An important feature for successful gene therapy is the efficient target to specific cells or tissues. An approach that is used to "solve" the problem of specificity that occurs, for instance with lentiviral vectors, is to change the tropism of its envelope by using a cell-specific ligand or scFv that recognizes and binds to specific cell receptors. Nevertheless, despite some level of specificity, there are problems concerning the low fusion activity and low viral titer. In an attempt to develop better delivering and targeting strategies, it has been used alphavirus envelopes like the one from Sindbis virus, to pseudotype lentiviral vectors, since this virus can be produced at very high titres, can achieve high levels of expression and can fuse with cells independently of the receptor binding protein. Nevertheless, even though effective *in vitro*, Sindbis virus has a nonspecific cell tropism *in vivo* because its receptors have wide distribution and are highly conserved. The insertion of a scFv and the generation of mutations in the Sindbis envelope glycoprotein would eliminate the problem of its non-specificity and has been shown, in fact, to augment the targeting strategies. However, these strategies might present some problems for *in vivo* applications, since there might occur non-specific reactions with plasma antibodies. Moreover, it needs cloning of a receptor specific antibody each time a new molecule needs to be targeted. To overcome these limitations it was developed a Sindbis pseudotyped lentiviral vector displaying a FITC scFv at the surface to target specifically any cell labelled with FITC-conjugated antibody. This new FITC scFv chimeric virus may have a wide therapeutic application, since any receptors or surface antigens can be targeted by this system. As a proof of concept of this strategy it was employed a model of T-cell leukaemia.

Therefore, the specific aims of this work were:

1) Expression of competent anti-FITC scFv at the surface of Sindbis envelope pseudotyped lentiviral vector;
2) *In vitro* specific targeting and killing of T-cells previously labelled with FITC-conjugated IgG;
3) Validation of this strategy *in vivo* in a leukaemia mouse model.

1. Introduction

1.1 Gene therapy: an overview

Gene therapy refers to the insertion of genes into an individual's cells or tissues to treat a disease, in which a defective gene (responsible for the disease) is replaced by a functional one, or is supplemented in the case it is absent. The main idea behind gene therapy has been the treatment of both inherited and acquired diseases but particular importance has been placed in targeting molecular processes associated with carcinogenesis and on improving gene transfer efficiency of current vector systems.

A large number of gene transfer protocols has involved *ex vivo* approaches, where cells are explanted from the patient, infected with a viral vector and implanted back into the patient. However, the long term goal is the development of *in vivo* delivery, i.e., to inject the patient with a vector capable of specifically delivering the therapeutic gene to a target tissue or cells.

Successful gene therapy depends on an efficient delivery and targeting of the therapeutic gene to specific cells or tissues in a safe way (without harming non-target cells) and with stable transgene expression. This would avoid problems with toxicity and unwanted healthy cells to be affected. An important factor to be considered is the virus native tropism. Often this should be diminished or ablated in order to avoid toxic side effects due to targeting to undesired sites. Vectors are usually engineered to target cells that they do not infect naturally but, for instance, in the case of herpesviruses its tropism can match its utility and can be therefore used for neuronal gene delivery [30].

Innate immune responses triggered by systemic vector administration can be a limitation of the use of gene therapy vectors. It is essential to overcome these vector-mediated innate immune responses, such as production of inflammatory cytokines, the maturation of antigen-presenting cells and tissue damage, because the induction of these responses not only shortens the period of gene expression but also leads to serious side effects.
When using viral vectors, the deletion of unnecessary viral genes considerably reduces cytotoxicity and immunogenicity and prevents the generation of replication-competent virus particles and subsequent spread of virus infection. Moreover, the transgene itself

can be highly immunogenic or even toxic to the host. Host immune response can be more problematic in the case of adenoviral vectors as they can elicit potent cytotoxic T cell responses against viral proteins that can eliminate the transduced cells. Moreover, because they do not integrate and are lost by cell division, they need repeated infection and the raised neutralizing antibodies will preclude repeated administration of the vector. In one of the early gene therapy trials in 1999, massive immune responses to an adenoviral vector used to correct a mutation led to the death of Jesse Gelsinger, a 18-year-old volunteer [200]. For instance, the main safety concern of using onco-retroviral vectors is related to the risk of malignant transformation following oncogene activation due to random onco-retroviral genomic integration. There is an apparent low risk of malignancy that is predominately associated with the occurrence of chronic retroviremia resulting from replication-competent retroviruses (RCR), particularly in immunosuppressed recipient hosts [225]. On the other hand, the strict dependency of onco-retroviral gene transfer on cell division is an important safety advantage that significantly limits the risks of horizontal transmission.

Successful clinical trials have been reported for hematopoietic diseases, such as X-chromosome-linked severe combined immunodeficiency (X-SCID) [33] and adenosine deaminase-deficient (ADA)-SCID. However, treatment of X-SCID with retroviral vectors has raised some safety concerns about its use in clinical gene therapy since the treated patients developed leukaemia[95], that seemed to be linked to the activation of a known oncogene adjacent to the vector insertion sites. To avoid or to repair this risk in the mean time, chromatin insulators can be used to reduce chances for retrovirus-mediated oncogenesis by inhibiting non-specific activation of close cellular proto-oncogenes [64]. Co-transduction of a suicidal gene under the control of an inducible promoter could also be one of the important safety measures, since destruction of transduced cells can be triggered if abnormal growth is observed. Additionally, conditional expression of the transgene only in appropriate target cells via the combination of targeted transduction, cell type-specific expression, and targeted local administration will increase the overall safety of the retroviral systems. Finally, splitting of the viral genome, use of self-inactivating (SIN) retroviral vectors, or complete removal of the coding sequences for gag, pol, and env genes is desirable to

virtually eliminate the possibility of generation of RCR [225]. Therefore, positioned genome integration and reduced cytotoxicity and immunogenicity are very important factors to take into consideration when choosing a gene therapy vector.

Ex-vivo transduction is better for introducing therapeutic genes into haematopoietic cells. For instance, in the case of congenital haematopoietic diseases, ex. X-SCID and ADA-SCID, delivery of therapeutic genes is achieved by isolating haematopoietic cells from patients, transducing it *in-vitro* and then re-infusing the cells back into the patients. In the case of cells of solid organs, it is difficult to isolate and replace these types of cells without causing the loss of their physiological functions and preservation of basic organ structure. One possibility for this would be to inject the gene therapy vector into the body, for example intratumoral administration, which result in transduction only in the injection surrounding area. Vector administration to the bloodstream would be a good mean to get to the target organs however, non-specific transduction may occur. Therefore, it is important to develop specific targeting strategies for an effective gene therapy approach.

1.2 Gene delivery vectors

An efficient delivery is crucial for the success of any gene therapy approach. Various viral and non-viral vectors have been engineered for improved gene and drug delivery.

1.2.1 Non-viral vectors

Although viral vectors have been widely used for years, it has been mainly the concerns about the safety and immunogenicity of the viral vectors that has driven investigators to develop non-viral vectors with targeting capacities for selective gene delivery. Non-viral vectors have low transfection efficiencies and lack of sustained gene expression and hence are little effective however, they have been improved to overcome these limitations and increasing attention has been given them because of advantages such as lack of immunogenicity, ease of preparation, and relative safety that, at the same time, make them suitable for repeated administration. Among these types of vectors are liposomes, cationic polymers, nanoparticles, naked DNA, antibodies and complexes of liposome with small-interfering RNAs (siRNAs) [172] or with single-chain variable fragments (scFv)[237] that can overcome the problem of non-specificity. Certain peptides, such as arginine peptides, containing high percentage of cationic amino acids can efficiently translocate through the cell membrane [99]. For instance, a nonamer arginine peptide (9R) conjugated to a anti-CD7 scFv was used for targeted delivery of siRNA into T cells enabling inhibition of HIV infection in a humanized mouse model[109].

Additionally, vectors that can replicate autonomously as an episome can be used as a delivery vehicle. For example, an Epstein-Barr virus (EBV) replicon non-viral vector (carrying mini-*ori*P) containing the diphtheria toxin subunit A (DT-A) was able to suppress tumour growth in a mouse mammary cancer model after electrogene transfer for *in vivo* transduction[196].

1.2.2 Viral vectors

Viral vectors have been the most used and the most efficient mean of gene transfer. Some viral properties, such as efficient delivery of nucleic acid to specific cell types

while avoiding immunosurveillance by the infected host, make them attractive gene delivery vehicles. The usage of a viral vector in gene therapy depends on factors such as packaging capacity, host range, cell-or tissue-specific targeting, replication competency, genome integration and duration of transgene expression [130](Table I). Therefore, its choice will depend on its ultimate application. For instance, in the case of cancer gene therapy, only high-level transient expression is required, while with genetic diseases it is required long-term therapeutic transgene expression.

The main groups of viral vectors used in gene therapy and their principal advantages or disadvantages are indicated in table I, to which should be added the alphaviruses. Viral vectors can be divided into two general categories: integrating and non-integrating. Integrating vectors include adeno-associated viruses (AAV), lentiviruses and retroviruses that allow long-term expression. Herpes simplex viruses (HSV) also allow for long-term expression since they cause a latent infection in the host. On the other hand, adenoviruses and alphaviruses give just transient expression levels. HSV have a strong tropism for neurons and large packaging capacity while the AAV do not allow the insertion of a large transgene. However, AAV are not inflammatory unlike adenoviruses that induce strong inflammatory responses, but are the most efficient in transducing the majority of tissues. The choice of the proper vector to apply in gene therapy should take into consideration the various features of each vector and the type of disease to be treated, as there is no ideal viral vector system available [130].

Table I. Viral vectors used in gene therapy. Adapted from [216]

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main limitations	Main advantages
Enveloped							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
HSV-1	dsDNA	40 kb* 150 kb‡	Strong for neurons	High	Episomal	Inflammatory; transient transgene expression in cells other than neurons	Large packaging capacity; strong tropism for neurons
Non-enveloped							
AAV	ssDNA	<5 kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenovirus	dsDNA	8 kb* 30 kb§	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

*Replication defective. ‡Amplicon. §Helper dependent. AAV, adeno-associated viral vector; dsDNA, double-stranded DNA; HSV-1, herpes simplex virus-1; ssDNA, single-stranded DNA.

Although efficient *in vitro,* in cell lines, viral vectors have not demonstrated the same efficiency *in vivo*. Innate and adaptive immune responses to vector particles and components are some of the limitations of gene therapy vectors that may restrict the efficiency of gene transfer and the persistent expression of the transgene. This can occur more often when using adenovirus based gene transfer systems. Nevertheless, vectors based on human adenovirus remain the most used gene delivery vehicles in clinic trials as they display *in vivo* stability and provide very efficient gene transfer to numerous dividing and non-dividing cell targets, without integration into the host genome, and are rarely linked to any severe disease in immunocompetent patients.

In addition, a number of vectors are derived from parent viruses that humans encounter through natural infection, resulting in pre-existing antibodies and possibly in memory responses against vector antigens. Similarly, an immune response can also be mediated against the transgene that has been delivered which may actually limit its sustained expression.

Other potential problems associated with viral vectors include the risk of insertional mutagenesis, difficulty in large-scale production, and size restrictions for exogenous DNA. These limitations of viral vector systems can significantly restrict their clinical application.

In comparison with non-viral vectors, the high packaging capacity of viral vectors is of great advantage when delivering large gene sequences. Moreover, the inclusion of an appropriate promoter/enhancer allows for specific regulation and increase in the levels of transgene expression. Viral vectors have also been developed to include a bidirectional promoter [5], or several promoters, making possible the simultaneous expression of multiple genes in the same cell. This feature allows, besides the inclusion of the therapeutic gene, the inclusion of a suicide gene, that can activate a pro-drug in case something goes wrong, or a reporter gene, which allow the monitoring of the efficiency of the gene therapy application.

Due to their capacity to stably integrate into the target cell genome, retroviruses have been quite used for gene therapy applications. However, its clinical use is currently limited by their poor efficiency in transducing non-dividing cells and the potential genotoxic consequences of their uncontrolled insertion into the human genome[12, 137].

1.2.3 Lentiviral vectors

Lentiviral vectors are derived from viruses belonging to the family of retroviruses (*Retroviridae*) and the lentiviruses genus. A typical structure of a retrovirus with its Gag, Pol and Env proteins is represented in Figure 1. Lentiviral vectors are usually generated by co-transfection of packaging cells with a transfer construct, a plasmid containing the vector genome, and the packaging construct encoding the viral proteins, that contains the trans-acting sequences, essential for assembly and function.

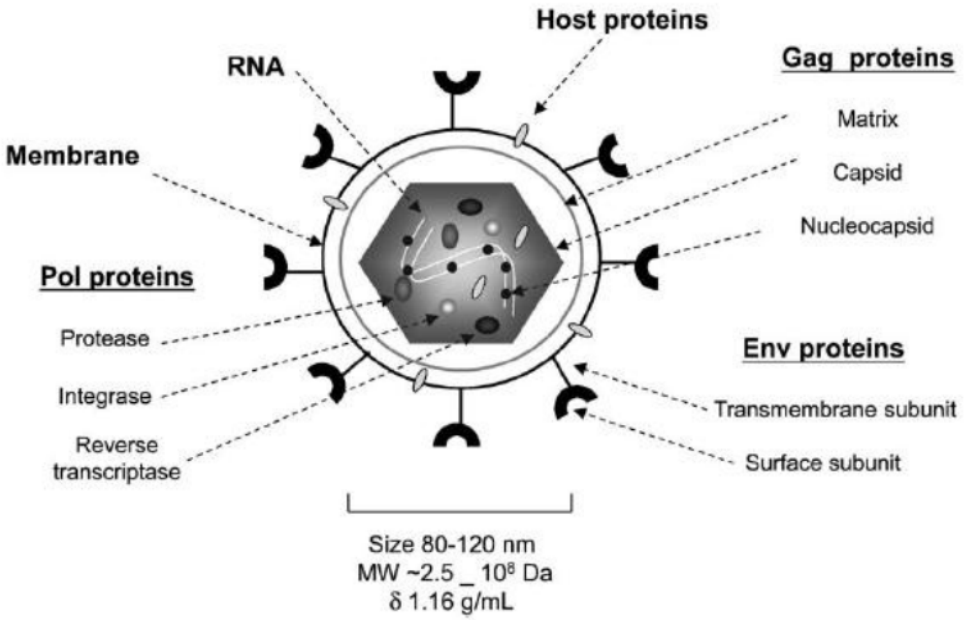


Figure 1. Retrovirus structure. Adapted from[193].

Lentiviral vectors have been widely used for gene delivery purposes. They can efficiently transduce non-dividing cells. The viral genome integrates into host chromosomes and the inserted gene can be maintained in the cells permanently. This capacity to maintain stable, long-term transgene expression, added to the considerable versatility in the design of the expression cassettes leading to improvements in term of

biosafety and efficacy, contribute to their increasing use, particularly in pre-clinical applications.

Because of the previous adverse events in clinical trials due to insertional mutagenesis using retroviral vectors as mentioned above, this is still an important point in vector safety. Nevertheless, lentiviruses, unlike gamma-retroviruses, are not associated with tumour development. Moreover, lentiviral vectors prefer to integrate in regions where expressed genes are concentrated[144, 192, 44], opposite to transcription start sites for retroviruses [234, 67]. To overcome this problem, integration-deficient lentiviral vectors have been developed[232]. Additionally, the integration of the transgene can be controlled using zinc-finger nucleases [32] and insulators[63]. As well, microRNA regulation can be applied to restrict transgene expression to a specific cell type by eliminating off-target expression in undesirable cells[28, 26].

Development of replication-competent lentiviruses (RCL) represents another safety concern as recombination between vectors and endogenous retroviral sequences could theoretically generate new human pathogens although, in this situation, non-human lentiviruses-based vectors could circumvent the problem as lentiviral infection is species-specific but, when a broad tropism envelope protein, like vesicular stomatitis virus-glycoprotein G (VSV-G), is used, this could be a major issue. Lentiviral vectors can be engineered to be safer and minimize the chances of generating RCL during vector production (Figure 2). The first generation of lentiviral vectors had the HIV envelope replaced by a heterologous envelope, such as VSV-G, in a different construct, which avoids formation of wild-type HIV (Figure 2D). Deletion of unnecessary viral genes from the packaging construct reduces cytotoxicity and immunogenicity and prevents the generation of replication-competent virus and spread of virus infection. This is characteristic of the second generation vector (Figure 2E). Removal of the transcriptional transactivator *tat* gene further improved safety and the additional separation of the *gag/pol* and *rev* gene expression into two non-overlapping expression constructs generates the third generation packaging plasmids (Figure 2F).

SIN lentiviral vectors are the ultimate generation of these vectors, in which the absence of long terminal repeat (LTR)-derived viral enhancers (in the 3' U3 region) and the reduced propensity to integrate a short distance from gene promoters gives it a better safety profile, as it reduces the risk of insertional oncogenesis, vector recombination and mobilization (Figure 2 B). The improved SIN includes sequences that allows efficient gene transduction of many cell types and enhances the levels of transgene expression (Figure 2C).

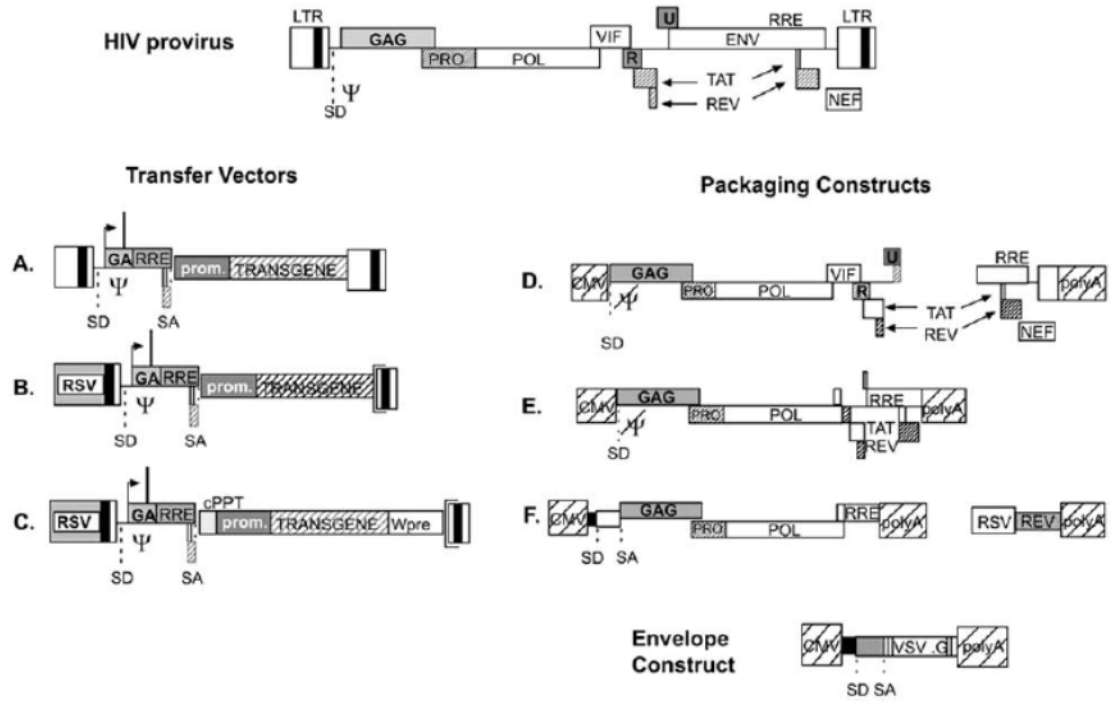


Figure 2. Schematic of HIV provirus, typical HIV-1-derived transfer vectors (left) and packaging constructs (right). (A) Wild type, **(B)** Self-inactivating (SIN), **(C)** improved SIN, **(D)** first, **(E)** second and **(F)** third generation packaging constructs. The envelope construct is unrelated to HIV-1 and is used to pseudotype the vector (E). **LTR**, long terminal repeats; **SD**, splice donor; **GA**, portion of the HIV-1 gag gene with a closed reading frame; **CMV**, cytomegalovirus strong promoter; **prom**., internal promoter; **polyA**, polyadenylation signal; **RSV**, rous sarcoma virus promoter; **SA**, splice acceptor; **Ψ**, packaging signal; **RRE**, Rev responsive element; **VSV-G**, vesicular stomatitis virus G protein; **WPRE**, post-transcriptional regulatory element of the woodchuck hepatitis virus; **cPPT**, central polypurine tract. Adapted from [163].

Although there is still some concerns regarding the safety of lentiviral vectors because few sequences are derived from HIV (~25%), they have been proven to be safe and there is no evidence suggesting that lentiviral vectors are capable of self-replication after infection. Indeed, HIV-1 derived lentiviral vectors are among the most efficient

and safest systems currently available for stable genetic modification of cells in culture. They actually have been the choice for researchers in the field of gene therapy.

In addition to HIV-1, lentiviruses vectors have been engineered mostly with Simian Immunodeficiency Virus (SIV), Feline Immunodeficiency Virus (FIV) or Equine infectious anaemia virus (EIV)[181, 224].

1.3 Immune responses to viral vectors and transgenes

The responses produced by the immune system can be not only against the vector but also against the transgene and these can have an impact on the therapeutic efficacy by restricting the effectiveness of gene transfer and the persistence of transgene expression. A gene therapy strategy for Duchene's muscular dystrophy employing a naked DNA gene transfer of the human dystrophy gene into *mdx* mice caused both a dystrophin-specific humoral and a cytotoxic T-cell response, even though human and mouse dystrophin proteins are more than 90% identical (some epitopes are different enough to trigger an immune response)[69]. There is a probability of occurring an immune response even if the difference between therapeutic and host protein is only a single amino acid [70]. When using allogeneic hematopoietic stem cell transplantation (HSCT) to treat hematologic malignancies there is a risk of occurring a graft-versus-host disease (GvHD) which is usually controlled by HSV-thymidine kinase (HSV-TK) suicide gene therapy that will induce a graft-versus-leukaemia immune response. In a clinical study, involving donor lymphocyte infusion with lymphocytes transduced to express HSV-TK, 7 out of the 23 patients treated developed a strong cytolytic T-lymphocytes (CTL) immune response against the HSV-TK protein, although in this case the immune response did not affect the efficacy of the therapy[217]. The transgene expression level could also be a determining factor. There were reports mentioning transgene-driven autoimmunity with AAV gene transfer of the erythropoietin gene[39, 79].

Tissue specificity can also influence the triggering of an immune response. That was the case when Follenzi and colleagues used lentiviral vectors for gene therapy of haemophilia B[71]. The expression of factor IX cDNA driven by a ubiquitously promoter (human cytomegalovirus, hCMV) triggered a specific cellular and humoral immune response which lead to clearance of the transduced cells and the transgene product but when the gene was expressed under the control of a liver-specific promoter that did not occur[71]. However, a recent study by Feng and colleagues[68] has shown that this does not always happen, as there is a large number of parameters that might interfere with the results.

Besides aspects related with the transgene, the vector used may also affect the host's immune responses. Innate and/or adaptive immune responses to transfer vectors can inhibit transgene expression (or eliminate transduced cells)[37, 229]. Viral vectors can stimulate the release of inflammatory mediators, cytokines, and chemokines [37]. This inflammatory response depends on the vector components (viral capsid, transgenes, marker genes), viral vector dose, site of injection, tissue injected, and cells transduced[122, 128]. Different vectors encoding a similar transgene, but injected into different tissues, can stimulate very different types of immune responses [90]. These responses are related, in part, with the viral tropism. For instance, the immunogenicity, and dissemination of adenoviral vectors to other cells or organs, limits the effective period of adenovirus-based gene therapy[186, 54]. However, these aspects will be overcome with improvements in vector design.

So far, there have been basically no reports on the potential of lentiviral vectors to induce inflammatory cytokine storm upon delivery into different tissues. However, there was a study showing that intravenous administration of late-generation lentiviral vectors in mice induced a rapid and transient interferon αβ response[27]. This inhibited transduction efficiency, specifically within the liver, and contributed to immune-mediated clearance of transduced cells. In a recent report, a doxycycline inducible lentiviral vector used to regulate the expression of erythropoietin led to an immune response against the tet-dependent transactivator (rtTA)[134]. However, that was not induced by the lentiviral vector as the same type of response was previously reported using a helper-dependent adenoviral vector harbouring the same type of transactivator to control long-term erythropoietin gene expression[114]. In a study characterizing the use of lentiviral vectors for central nervous system (CNS) gene therapy no measurable inflammatory responses were shown after vector injection. Subsequent systemic immunization with a lentiviral vector carrying the same transgene as the vector injected into the CNS resulted in a decrease in transgene expression and inflammation that were caused by the immune responses against the transgene, since with a lentiviral vector carrying a different transgene no immune responses were observed. This demonstrated the low immunogenicity of lentiviruses and prolonged transgene expression even in the presence of pre-existing lentiviral immunity[1]. Nevertheless,

although lentiviral vectors are less immunogenic than other viral vectors, they can elicit certain immune responses *in vivo*, limiting their clinical application in gene delivery. They have been shown to elicit strong CTL responses against the transgene-encoded proteins what make them excellent vectors for anti-tumour immunotherapy[61, 21].

VSV-G is largely used in lentiviral vector preparations but there are some aspects that need to be taken into consideration. A few studies showed that VSV-G-pseudotyped LVs are inactivated by human serum complement[53], although the incorporation of complement regulatory proteins have shown to provide complement resistance[187], and the *in vivo* use of unaltered VSV-G-pseudotyped vectors in human patients would be problematic because of complement inactivation. In addition, VSV-G-pseudotyped lentiviral vectors can form tubulovesicular structures in transfected cells that carry residual amounts of the plasmid DNA used for transfection, which can be co-purified with viral particles during viral concentration, and therefore act as strong activators of plasmacytoid dendritic cells that induces the secretion of high levels of IFN-α[170].

Serum complement inactivation is also one of the major obstacles in the use of baculovirus vectors for *in vivo* gene transfer. By displaying complement regulatory proteins on its surface, such as decay-accelerating factor (DAF), it was possible to protect the vector against complement inactivation and reduce inflammatory responses[94].

The amount of viral vector and its quality can also be an issue. Suboptimal vector manufacturing or excessive dose increase may trigger toxic and inflammatory nonspecific responses after intravenous administration. Therefore, vector production and purification can be optimized using new protocols that can avoid or minimize unexpected immune reactions, toxicity or inflammation due to contaminants. For instance, purification by sucrose gradient ultracentrifugation was reported to abolish the immune response, however vector titers also decreased considerably[10]. On the other hand, lentiviral vector production in the absence of serum in the cell culture

medium reduced immunogenicity in the same way but without affecting transduction efficiency[10].

1.4 Targeting strategies

The development of efficient, safe and cost-effective clinical applications will depend on a better ability of delivery vectors to target specifically the cells of interest. Currently, three types of strategies can be applied for targeting lentiviral vectors. These consist in: 1) targeting at the level of vector-cell entry through lentiviral vector surface modifications; 2) targeting at the level of transgene transcription by insertion of tissue-specific promoters into lentiviral vectors; 3) a novel microRNA technology that rather than targeting the 'right' cells will 'detarget' transgene expression from non-target cells, while achieving high expression in the target-cell [75].

Viral vector tropism can restrict the application of gene therapy, as vectors are directed towards specific receptors on the cell surface. A mean to overcome this is to substitute the endogenous envelope glycoprotein for another one that will broaden that tropism. Specific tissue tropism can be achieved by altering the envelope protein, which can be done by (Figure 3): i) engineering onto the envelope a ligand for a receptor expressed on the target tissue, ii) engineering onto the envelope an antibody binding site that recognizes a cell-specific antigen on the target tissue and iii) pseudotyping.

The advantage of an antibody over a ligand is that it acts as an antagonist for a given receptor, whereas its ligand will inevitably elicit a biological response upon binding (triggering a signalling pathway).

Regarding transcriptional targeting, the existence of tissue-specific (or cell-specific) and tumour-specific enhancer/promoter sequences that can drive the expression of therapeutic genes can be a benefit to targeting[242]. Nevertheless, this kind of promoters are not as stronger as viral promoters (such as CMV), or other ubiquitous promoters (such as UbiC), and as a result, gene expression and consequently transduction efficiency are reduced.

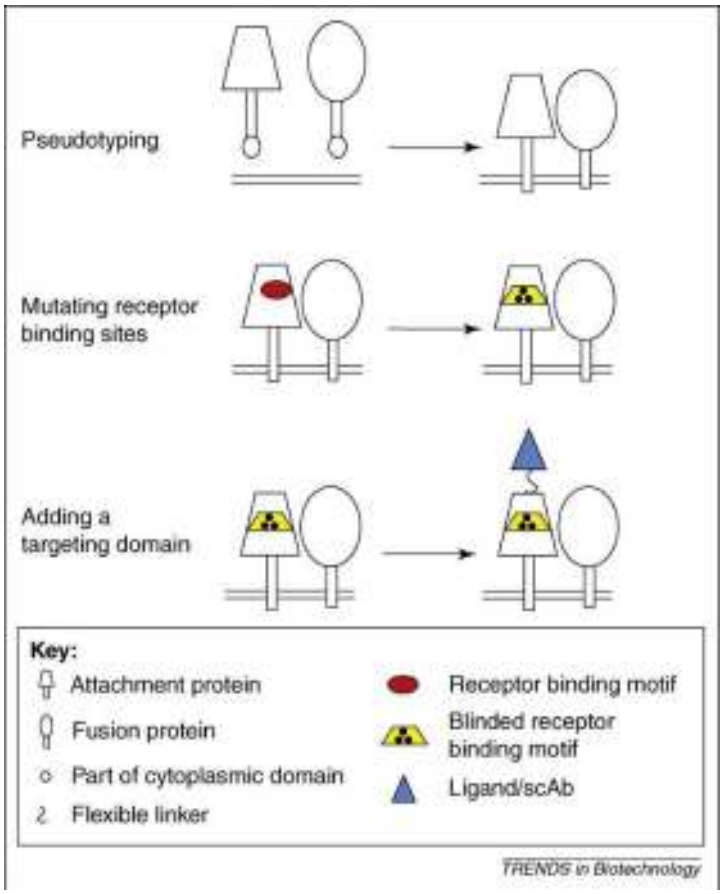


Figure 3. Principle of re-targeting of lentiviral vectors. Adapted from [29].

1.4.1 Pseudotyping lentiviral vectors

Usually, the host-cell tropism of a virus can be changed by replacing the virus attachment protein with that of a related virus, a method called pseudotyping. Pseudotyping was first described when investigators noted that host cells infected with two enveloped viruses generated progeny, with both the native envelope and the co-infected viral envelope [40].

Although pseudotyping can be used for non-enveloped vectors like AAV and adenoviruses, it has been used most extensively to modify the tropism of retroviral (and lentiviral) vectors because they are highly permissive for incorporation of heterologous attachment glycoproteins [49]. For this incorporation to occur, it is important a high cell-surface expression level in the packaging cell at the site of virus budding[184]. Additionally, some modifications in the cytoplasmic tail of the pseudotyping glycoprotein might also be necessary[41, 140], as interactions between them

and the components of the virion core dictate assembly of viral particles for some incorporation models.

Viral entry of enveloped viruses occurs in a two-step process. First, the viral surface protein binds to receptors on the plasma membrane of the host cell. After that, membrane fusion takes place between the lipid bilayer of the viral envelope and host cell membranes. This fusion reaction can occur either at the plasma membrane (direct entry, pH-independent) or in acidic endosomes following receptor-mediated endocytosis (indirect entry, pH-dependent). In pH-independent cell entry, the fusion between the viral envelope and the cellular membrane occurs directly at the cell surface. During direct entry at the cell surface, the membrane fusion process is triggered by receptor contact that induces conformational changes in the glycoprotein. During the endocytic process, the low pH environment induces structural rearrangements in the glycoprotein that leads to membrane fusion. In either case, the membrane fusion reaction delivers the viral nucleocapsid into the host cytoplasm. The envelope glycoproteins of VSV[210], human foamy virus[168], Ross River virus (RRV) and other alphaviruses[98] are some of the examples involved in pH-dependent cell entry. On the other hand, viruses such as HIV-1[204], the feline retrovirus RD114[136] and measles virus[199] use a direct viral entry.

Lentiviruses are membrane-bound virions that acquire their membrane immediately prior to being released from the host cell. Therefore, pseudotyping is facilitated since the envelope glycoprotein, that is important in determining tissue tropism, is not required for budding, as the viral core particle can form and bud in the absence of any glycoprotein[171]. Any transmembrane proteins that are present at the site of budding become incorporated into the lentiviral vector particle and are displayed on its surface [36], although some envelope glycoproteins assemble with lentiviral core at late endosomes[184].

There is a long list of foreign envelope glycoproteins that have been incorporated into lentiviral vectors (see the paper by Bischof and Cornetta[18] for a list of glycoproteins successfully used for pseudotyping). A major contribution to the advances in lentiviral

vector application for gene delivery was the efficient use of VSV-G to pseudotype HIV-1 virions[153] that, together with fact that it allows infection of a broad range of cell types in many species, made it the most used glycoprotein for pseudotyping. Nevertheless, this broad tropism of VSV-G can be problematic when tissue specificity or cell targeting is required. VSV-G has other disadvantages including cytotoxicity[178], potential for priming of immune responses against transgene products through efficient transduction of antigen-presenting cells (APCs)[101], such has dendritic cells, and sensitivity to inactivation by human complement [53]. For these reasons, other envelopes have been used as an alternative to VSV-G to pseudotype lentiviral vectors.

Although many viral envelopes have been used to pseudotype HIV-1-derived lentiviral vectors[18], the infectious titres are usually low when compared with VSV-G pseudotyped lentiviruses. On the other hand, SIV-derived vectors pseudotyped with amphotropic or modified feline endogenous retrovirus envelope (RD114/TR) transduced more efficiently human and macaque primary blood lymphocytes and $CD34^+$ cells than the vector pseudotyped with VSV-G[183]. Additionally, RD114/TR-pseudotyped vectors may be concentrated by ultracentrifugation and are resistant to complement inactivation[183]. Moreover, a study comparing VSV-G-pseudotyped vectors with RD114/TR-pseudotyped particles showed that the chimeric envelope was able to transduce hematopoietic stem/progenitor cells at a lower multiplicity of infection (MOI), with less toxicity and pseudo-transduction at comparable vector copy number *per* genome[56].

Ross River virus-pseudotyped HIV-1-derived lentiviral vectors were able to transduce epithelial and fibroblast-derived cell lines from various tissues at levels comparable to VSV-G[92], while in another study by the same research group, transduction of hematopoietic cells was significantly impaired[93], which at the same time can be an advantage in clinical applications when targeting of hematopoietic tissue should be avoided.

The choice of the envelope glycoprotein for pseudotyping depends on the tropism that needs to be achieved, i.e., a broad or a more restricted one. There is a limited

availability of viral envelopes with a natural restricted tropism, and for that reason, most of the envelope glycoproteins used for pseudotyping are the ones with a broad tropism. Nevertheless, there have been some reports describing the use of the former ones. For instance, the Mokola virus envelope protein was used for astrocytes transduction [45], the glycoprotein from lymphochoriomenigitis virus was used for glioma cells targeting [143], the glycoproteins from measles virus wild-type strain for activated human lymphocytes [78] and also the glycoproteins from HIV that targets specifically CD4-expressing cells [191, 124, 215]. This means that a certain envelope from a virus with a tropism for a specific cell-type or tissue might be used for the treatment of a disease in which those cells or tissues are affected. For instance, the use of envelope glycoproteins from virus infecting the lungs via the airway epithelia can be useful for vector pseudotyping in gene therapy for diseases of the respiratory tract[103]. Also, Murine leukemia virus (MLV) pseudotyped with human hepatitis B virus (HBV) large (L) and small (S) surface antigens (HBsAg) exhibits strict tropism for primary human hepatocytes, similar to the natural target cell specificity of HBV, thus offering a potential liver-specific targeting system for gene therapy [209].

However, vector pseudotypes may not always retain the same tropism as the parental virus from which the glycoproteins were derived, as occurred for instance with neurotropic Mokola virus glycoprotein pseudotyped HIV-1 based vector[55].

Sometimes it might be required to truncate the cytoplasmic domains of the glycoproteins used to pseudotype the viral vector in order to get proper envelope function. For instance, RD114/TR contains the RD114 cytoplasmic tail of its glycoprotein replaced for that of MLV-A[183, 56]. This chimeric glycoprotein preserve the host range of the original RD114 and have increased titres compared to SIV vectors pseudotyped with the wt glycoprotein[183]. Others have also substituted with success the cytoplasmic tail of the parental envelope glycoprotein by that of MLV[206, 41].

The engineered glycoproteins must fold correctly, be stably incorporated on virions and allow efficient retargeted virion binding to the expected cell-surface molecules. Although the majority of the exploited ligand-displaying envelope glycoproteins can

bind specifically and efficiently to the targeted cells, the infectivity of the viral particles is usually low. Indeed, infectivity of the recombinant viruses can be inhibited at a post-binding step, as occasionally the chimeric glycoprotein incorporated on viral particles maintains the fusogenic potential but the interaction of the displayed ligand with its target cell-surface molecule is generally not able to activate the fusion functions of the chimeras[243]. Besides this inability to induce membrane fusion (inability of the targeted receptors to activate envelope fusogenicity), the sequestration of the targeted receptor-bound retroviral particles to cell compartments by some types of cell-surface molecules might also abolish infection[48]. When aiming at retargeting a specific surface receptor molecule, the receptor attachment function in an envelope protein has to be altered, whereas the membrane fusion function has to be kept completely active. Thus, to circumvent the above-mentioned constraints, these two functions have been recently separated in two proteins[120]. This has been successfully used for instance for Sindbis envelope glycoprotein[239, 76, 113, 111].

Other envelopes that have been widely used lately are those from Sindbis virus and Measles virus because of their ability to incorporate a ligand, an antibody or a scFv for efficient targeting. Funke and colleagues have obtained specificity and high transduction efficiency by altering the measles virus envelope to preferentially infect $CD20^+$ cells to target lymphoid tissue[77]. Moreover, in a different study by the same group, they targeted different cell surface molecules on different cell types taking advantage of scFv antibodies for those molecules and where natural receptor usage was prevented by mutation of the relevant residues in the hemagglutinin protein receptor recognition domain[6]. Recently, measles virus hemagglutinin (H) and fusion (F) envelope glycoproteins were used to pseudotype replication defective VSV and redirect entry and infection specifically to tumor-associated receptors, through the display of scFv antibodies specific for epidermal growth factor receptor (EGFR), folate receptor (FR) or prostate membrane specific antigen (PSMA)[8].

Special attention will be given to Sindbis virus envelope in the next section.

1.4.2 Sindbis pseudotyped lentiviral vectors

Sindbis virus is an enveloped virus with single-stranded positive sense RNA genome. It is the prototype of the alphavirus genus in the Togaviridae family [208]. The Sindbis genome codifies for non-structural and structural proteins but only the structural proteins, namely capsid (C), E3 peptide, envelope protein 2 (E2), 6K peptide and envelope protein 1 (E1), are present in the Sindbis pseudotyped lentiviral vectors. E2 protein mediates interactions with the target cell receptors, laminin receptor and heparin sulphate[230, 31, 102], and E1 mediates low pH-dependent fusion. Unlike retroviruses, the Sindbis virus fusogenic E1 protein can fuse to cells independently of the receptor binding E2 protein. Modified retroviral envelope proteins, despite having specific binding activities, have low fusion activity resulting in inefficient entry into cells [243]. The Sindbis envelope proteins mediate binding to host cell receptors, leading to the endocytosis of the virion. When the endocytic vesicle is acidified, the envelope proteins undergo conformational changes that result in the fusion of the lipid bilayer of the virion and that of the vesicle. The nucleocapsid, a complex of the capsid protein and the genomic RNA, is then deposited into the cytoplasm of the host cell[91, 117].

Sindbis virus has a wide host-range because its host-cell receptors, laminin and heparin sulphate are widely distributed and are highly conserved [208], resulting therefore, in an *in vivo* non-specific infection. But apart from this, it gives a high level of gene expression, it can infect non-dividing cells and can be purified to high titers[236, 208], which makes this virus an efficient vector system for delivery and expression of fusogenic membrane glycoproteins.

Sindbis virus is an oncolytic virus that selectively targets tumours through the laminin receptor, which is overexpressed in these cells[220, 83]. In fact, downregulation of this receptor reduced Sindbis vector infectivity, further confirming its role in mediating the tumour-targeting ability of Sindbis[219]. In a study by Scheiman and colleagues[188], Sindbis-pseudotyped lentiviruses expressing a short-hairpin RNA (shRNA) specific for the 37/67-kDa laminin receptor (LAMR) could target and inhibit tumour growth via binding and downregulation of that receptor showing once more its importance for cancer gene therapy. Besides this, the non-structural proteins of Sindbis are potent

inducers of apoptosis and thus, it naturally induces cell death in tumour cells. In addition, the blood-borne nature of Sindbis enables Sindbis vectors to reach tumour cells throughout the body. For all these reasons, it has been used mainly in cancer gene therapy. In a report, Tseng and colleagues demonstrated that a single intraperitoneal treatment allowed a Sindbis vector to target systemically and eradicate tumour cells throughout the body without adverse effects [221]. Another study by the same group, showed that Sindbis vectors containing a HSV-TK gene could successfully deliver this suicide gene to tumour cells that in combination with the pro-drug ganciclovir (GCV) exposure provided subsequent GCV activation and tumour killing [222]. Therefore, these studies indicate that the incorporation of reporter genes in Sindbis vectors provides a mean of tumour detection and subsequent suppression.

Vectors based on the Sindbis virus RNA genome were constructed where the E2 envelope protein was modified by insertion of an Fc-binding portion (ZZ domain) of *Staphylococcus aureus* protein A [159] and demonstrated to be able to target efficiently human cell lines when used in conjunction with monoclonal antibodies. But being a lytic RNA virus, Sindbis virus is not suitable for applications requiring stable transduction [87]. Therefore, Morizono and colleagues used instead HIV-1- and murine leukaemia virus-based retroviral vectors pseudotyped with a modified Sindbis virus envelope containing the Fc-binding domain of protein A to target specific cells *in vitro* [147]. However, there is non-specific gene transduction to the liver and spleen with this pseudotyped virus. This problem was overcome with the generation of a modified Sindbis envelope containing mutations in specific domains of E2 and E3 glycoproteins [152]. These strategies based on the incorporation of the ZZ domain into the envelope glycoproteins have been widely used[16, 152, 4, 105]. However, it might cause problems after systemic administration due to competition for binding between the antibodies present in the serum and the targeting antibody. Additionally, other limitation of this ZZ system that might also affect the targeting efficiency is the non-covalent linkage to the antibody.

Lentiviral pseudotyping with Sindbis virus envelope has been widely used and is a great promise for gene delivery systems. Sindbis envelope protein pseudotyped

lentiviral vector displaying anti-CCR5 scFv lead to specific targeting to CCR5-expressing cells and primary lymphocytes *in vitro* [2]. P-glycoproteins on metastatic melanoma cells in lung tissue were also successfully targeted by modified lentivirus pseudotyped with a chimeric Sindbis envelope (termed m168 and lacking nonspecific infectivity) and a surface displayed anti-P-glycoprotein antibody, through intravenous injection [152]. Additionally, by incorporation of an antibody conferring target specificity and a modified influenza hemagglutinin mutant mediating pH-dependent membrane fusion, the pseudotyped lentiviral vector was successfully used to target CD20 in human B cells *in vitro* and in animals [240]. The incorporation of biotin-adaptor peptides or integrin-targeting peptides into the envelope of Sindbis-pseudotyped lentiviruses were also successfully employed for redirecting vectors to the specific receptors via conjugation with a targeting molecule or antibody[149, 151].

More recently, this method of lentiviral pseudotyping with engineered Sindbis envelope was reported for immunization against cancer, where dendritic cells were targeted specifically *in vivo* through its cell surface molecule DC-SIGN thereby inducing strong antigen-specific immune responses [241].

1.4.3 Single-chain antibodies (scFv) as a targeting vehicle in gene therapy

As already mentioned, viral envelope glycoproteins mediate the binding and fusion between virus and target cell, allowing the control of target cell recognition and host range of the viral vectors used for gene delivery. Then, when one wants to target a specific surface receptor molecule for cell entry, the way is to engineer the envelope glycoproteins (re-targeting) and usually it is the domain of the envelope protein responsible for receptor binding that is modified. Therefore, inserting a ligand, scFv or antibody bridge into this structure allow their display at the virus surface and therefore the specific targeting of cells.

Single-chain antibodies are intracellular antibodies (intrabodies) that consist of the variable domains of the heavy (VH) and the light (VL) chain of an antibody connected by a linker peptide. They have the advantage of being highly specific and possess a high affinity for the target and for that reason, they have increasingly been used in the field of gene therapy to target intracellular molecules with potential therapeutic applications. For example, this was done some time ago by the group of Dr. Roger Pomerantz in which they inhibited the early stages of HIV-1 life cycle by intracellular expression of scFvs against integrase and reverse transcriptase in order to control HIV-1 infection[115, 195]. Other important characteristics and usages of intrabodies that can be very advantageous to gene therapy applications include redirecting target antigen to a particular subcellular location through an appropriate trafficking signal peptide fused with it and the unique ability to specifically disrupt a specific function of a multifunction protein[11, 47]. ScFvs have been used also to target infected cells. For instance, scFvs against the laminin receptor were used in an *in vivo* gene delivery system, based on AAV vectors, to reduce prions propagation[246].

The use of scFv in gene therapy has gained increased interest in the last several years. One of the first studies to describe the use of scFvs to target retroviral vectors specifically to human cells was the work by Somia and colleagues[201]. They fused a scFv directed against the low density lipoprotein receptor to the envelope of Moloney murine leukaemia virus (MoMLV) and infected specifically the cells through the recognition of that receptor. In particular, scFv have been most used in the cancer

research field to target molecular processes closely associated with carcinogenesis, being the oncogenes the more frequent molecular targets for scFv. Recently it was demonstrated the inhibition of LMO2-dependent leukaemia in a mouse T-cell tumourigenesis transplantation assay with retroviral-mediated expression of anti-LMO2 scFv[154]. LMO2 is an oncogene whose insertional activation was associated with the development of leukaemia in patients involved in an X-SCID gene therapy clinical trial. The oncogene c-erbB-2 was also efficiently targeted by an adenovirus engineered to express an adapter protein containing a scFv against c-erbB-2[96]. The authors demonstrated that the adaptor protein efficiently blocked adenovirus native tropism while simultaneously mediating virus infection, and thus enhanced gene transfer efficiency to cancer cell lines overexpressing c-erbB-2[96]. Additionally, amphotropic retroviruses with modified envelope displaying scFv directed against the c-Met receptor were generated and found to efficiently and selectively deliver genes into hepatocarcinoma cells[156].

This strategy of targeting through scFv display has been previously used in Dr. João Gonçalves lab to successfully target cells expressing the CCR5 chemokine receptor, as already mentioned above[2].

Alternatively, in the case of viral vectors without envelope, like adenovirus, the scFv can be fused to a capsid protein. Efficient and functional incorporation of a fusion between hyper-stable scFv (directed against beta-galactosidase) and adenovirus minor capsid protein IX (pIX) into the adenovirus capsid was reported by Vellinga and colleagues as a possible strategy for retargeting[226], thus demonstrating that pIX can also be used as a platform for the presentation of scFv antibodies.

Still, the inclusion of a scFv is not always favourable. The insertion of a scFv targeted to folate-binding protein (FBP) into the N-terminus of MoMLV Env resulted in the reduction of the infectivity and the kinetics of entry of the MoMLV vectors. The scFv targeted to FBP increased the threshold for fusion and might have re-routed the entry of the targeted MoMLV-FBP vector towards an endocytic, non-productive pathway[227]. In addition, despite scFv could be displayed on the capsid of adenovirus through

genetic fusion to native pIX, these molecules failed to retarget the virus, due to improper folding of the scFv. This could be overcome with single-domain antibodies (sdAb)[173].

As mentioned earlier, previous studies based on the addition of ligand motifs that binds to specific molecules associated with the cell membrane allowed binding to the new receptors and internalization, but the infection titres were low. Inefficient transduction was mostly due to diminished fusion activity of the engineered glycoprotein, most likely because this chimeric protein cannot undergo the appropriate conformational change that is thought to be triggered by receptor binding and that lead to viral entry[13, 243]. An attempt to redirect cell transduction was also performed for the widely used VSV-G. A large ligand binding domain, a scFv against major histocompatibility complex I (MHC-I), was attached directly to the N-terminal of the glycoprotein[58], a site previously shown to be permissive for insertion of short peptides [189]. Although this provided a new binding specificity, cells were transduced very inefficiently, again probably due to inefficient fusion activity. More recently, this fusion inability has been overcome by inclusion of a fusogenic protein in the engineered chimeric envelope. The laboratory of Dr. Pin Wang has developed a method to incorporate a membrane-bound antibody (anti-CD20) and a fusogenic molecule derived from Sindbis virus glycoprotein to provide binding and fusion functions respectively, into gamma-retroviral vectors for targeted gene delivery[239].

Gene constructs expressing scFv may also be incorporated into non-viral vector systems. For example, by linking nucleic acid-binding human protamine to the C-terminus of an anti-erbB2 scFv antibody[118], exogenous DNA could be selectively delivered into erbB-2 positive cells. Alternatively, a liposome, which can hold within its lipid bilayer nucleic acids or proteins, has been coupled with antibodies (immunoliposome) to facilitate targeting and endocytosis to specific cells[157].

ScFv have also been used in siRNA delivery. An ErbB2 single-chain antibody fused with protamine delivered siRNAs specifically into ErbB2-expressing cancer cells and the same report describe the use of a protamine-HIV-1 envelope antibody fusion to

deliver siRNA only to cells expressing the HIV-1 envelope[202]. In a work by Kim and colleagues, two antibody-based strategies were developed for systemic delivery of siRNA for anti-HIV-1 therapy, either specifically to T cells via the CD7 receptor or to multiple immune cell types via LFA-1, present on all leukocytes, using a cationic-peptide based delivery or a liposome-based delivery, respectively[100].

1.5 Suicide gene therapy

In suicide gene therapy, the gene encoding an enzyme is delivered to target cells, followed by administration of a prodrug, which is converted locally to a cytotoxin by the enzyme. Consequently, the target cells, as well as surrounding bystanders, are killed. Investigation in suicide gene therapy has been mostly applied for the targeting of cancer cells and essentially as a safety control of the therapeutic procedure. When replication-competent viral vectors (or oncolytic virus) are used, one safety measure employed to reduce the risk of toxicity associated with possible vector propagation to normal tissue cells is to include a suicide gene into one of the non-structural genes assuring in that way its co-expression. This was done in a study by Tseng and colleagues[218] using a simple replication-competent (RC) Sindbis viral vector where they show a considerable increase in *in vivo* tumour targeting and killing capability of that viral vector/suicide gene system comparing with the conventional replication-deficient vectors. Another widely used application of suicide gene therapy is in gene therapy for allogeneic hematopoietic stem cell transplantation, a therapeutic modality for patients affected by haematological diseases. A major complication in these disorders is the incidence of graft-versus-host disease where alloreactive donor T cells recognize host antigens presented by recipient cells. The suicide gene therapy is employed in these situations to exploit the alloreactivity against malignant cells[131]. Importantly, the activation of the suicide gene does not interfere with the process of reconstitution of the immune cells by the graft[42].

Some of the most common suicide gene therapy systems used include the already mentioned HSV-TK/GCV, the *Escherichia coli* cytosine deaminase/5-fluorocytosine (CD/5-FC), and the suicide genes inducers of cell death, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) and factor-related apoptosis ligand (FasL or CD95L). The CD/5-FC suicide strategy was shown to be associated with the triggering of cellular and molecular events leading to an efficient antitumor immune response involving both innate and acquired immunity, in a rat liver metastasis model[17]. An scFvC45: sTRAIL antigen has been used to target specifically EGP2 antigen on the surface of tumour cells and induce apoptosis[22, 23]. TRAIL has also

potential for application in allogeneic hematopoietic-cell transplantation for an enhanced graft-versus-tumour effect[190]. The apoptosis-inducing activity of scFvCD7: sTRAIL was even stronger than that of the immunotoxin scFvCD7: ETA[24]. The same group of investigators have developed a fusion protein, designated scFvCD7: sFasL that consists of soluble FasL genetically linked to a high-affinity scFv antibody specific for the T-cell leukaemia-associated CD7 antigen. Soluble homotrimeric scFvCD7: sFasL is inactive and acquires tumoricidal activity only after specific binding to tumour cell-surface-expressed CD7[25].

Nevertheless, HSV-TK is the prototype of suicide gene therapy. This enzyme can phosphorylate the nucleoside analogue GCV, which can inhibit cellular DNA replication by substitution for normal nucleosides in the DNA chain leading to premature interruption of replication and cell death. Although this system is efficient and safe in the selective control of GvHD, it has some disadvantages. The first relates to the presence of an active cryptic splicing site in the TK gene, resulting in a non-functional TK[80]. Therefore, some mutant variants were developed to overcome the problem with alternative splicing[34] that is important to ensure high GCV sensitivity to transduced target cells. Fehse group constructed a fully codon optimized mutant HSV-TK suicide gene that combines better killing performance with reduced unspecific toxicity[175, 174]. The second limitation is concerned to immunogenicity of viral TK in humans leading to the unwanted elimination of gene-modified cells [180]. This is, in fact, a problem in immune reconstituted patients15 but not in the immunodeficient ones[217, 139].

Other suicide genes currently used provide an alternative to HSV-TK, as they are not immunogenic in humans or have a low immunogenicity. CD20, a cell surface molecule, is the first human suicide gene developed where anti-CD20 monoclonal antibodies are used to kill $CD20^+$ cells. It has also the advantage of being used as a selection marker[88]. CD20 has also been applied as a novel suicide gene system particularly for the treatment of GvHD. In a study by Serafini and colleagues, exogenous CD20 protein was used both to mediate selection of transduced human T lymphocytes (using a Moloney-derived retroviral vector) and subsequent killing with

rituximab, a commercial available antibody against CD20[194]. Additionally, Griffioen and co-workers[81] also reported a successful application of this suicide gene strategy in adoptive T-cell therapy of cancer.

A novel suicide system that can also overcome the immunogenicity of the use of HSV-TK is the chimeric inducible caspase 9 (iCasp9) that is based on the fusion of truncated human caspase 9 to a modified human FK-binding protein, allowing conditional dimerization. When exposed to a synthetic dimerizing drug, Casp9 becomes activated leading to the rapid death of the cells in which it is expressed. This strategy has been used as a safety switch in adoptive cell therapies[214, 52, 57].

Other genes that have been used in suicide gene therapy are those from bacterial toxins, such as *Pseudomonas aeruginosa* exotoxin A (ETA) and DT-A. Both toxins are very potent inhibitors of protein synthesis and catalyze ADP ribosylation of human elongation factor 2 (EF-2), which triggers cell death by apoptosis. It has been estimated that a single molecule of DT is sufficient to kill a cell[238]. DT (and ETA) has two subunits, the B subunit which is responsible for binding to the cell and the A subunit which contains the catalytic domain of its enzymatic activity. In particular, numerous human clinical trials have been conducted using DT-fusion proteins for cancer therapy, for instance in patients with previously treated chronic lymphocytic leukaemia (CLL), i.e., with recurrent or refractory CLL[73, 74].

Indeed, treatment of tumours with cytotoxic agents coupled to antibodies or ligands directed to tumour cell-specific structures is truly promising as demonstrated by numerous clinical studies and approved drugs such as Ontak, a fusion between peptide sequences of diphtheria toxin and human IL2[62]. The immunotoxins, molecules that contain a protein toxin and a ligand, have been applied in the treatment of cancer, particularly in chemoresistant hematologic malignancies[107]. A malignancy where recombinant immunotoxins are most successfully applied is hairy cell leukaemia (HCL). A good example is the fusion of a truncated form of *Pseudomonas* exotoxin A (termed PE38) with the variable domain (Fv) of anti-CD22 antibody[108, 107] that has been further improved to reduce immunogenicity[160].

1.6 Applications of gene therapy in diseases and in clinical trials

The field of gene therapy has made important advances in the last decades that has made possible to move from the laboratory research to the clinical trials on a diversity of diseases.

It is possible to find a wide variety of gene therapy applications that are currently in clinical trials or that have already terminated, within both the US and other countries, through the website ClinicalTrials.gov. Remarkably, almost all of the registered trials involving viral vectors employ AAV or Adenoviruses. Accounting for that, in addition to the previously mentioned advantages, are the well established production parameters for clinical grade Adenoviruses and AAV vectors.

Currently ongoing are several human gene therapy clinical trials to evaluate the use of lentiviral vectors for treatment of human diseases such as Parkinson's disease, β-thalassemia, X-linked adrenoleukodystrophy (X-ALD), and acquired immunodeficiency syndrome (AIDS). The improvements in lentiviral vectors design already mentioned, mainly those regarding safety, have important implications for its adoption as the vector of choice for clinical trials. Indeed, the first lentiviral vector on phase I clinical trial was a HIV-1-based vector carrying an antisense sequence against the HIV-1 envelope gene. Transduction of $CD4^+$ T lymphocytes with this vector (VRX496) resulted in expression of the therapeutic antisense sequence and subsequent inhibition of productive HIV-1 replication. The main objective of the study was to determine the safety and tolerability of treatment with autologous $CD4^+$ T cells modified (transduced) *ex vivo* with the vector VRX496 when administered to HIV-infected patients[132]. This vector is already in a phase II, open-label, multicenter study to evaluate the safety, tolerability, and biological activity of single and repeated doses of autologous T cells transduced with the vector in HIV-positive individuals (Clinical trials identifier number NCT00131560).

Some of the most important advancements in gene therapy for diseases include genetic disorders such as ADA-SCID; chronic granulomatous disease (CGD) and haemophilia,

to which exciting treatment results have been obtained in appropriate animal models of the disease. In ADA-SCID, affected children are born without an effective immune system and will succumb to infections without bone marrow transplantation from matched donors. *Ex vivo* gene therapy with retroviral-transduced $CD34^+$ bone marrow cells was applied to deliver the therapeutic ADA gene to the patients. The immune system was reconstituted in all treated patients without noticeable side effects, and without need for further treatment[3] (ClinicalTrials.gov NCT00598481 and NCT00599781). CGD is a genetic disease affecting the immune system that leads to the patients' inability to fight off recurrent bacterial and fungal infections, leading to the formation of chronic granulomas that can be life threatening. Two patients with this disease were treated in a gene therapy trial. However, after initial resolution of the infection, the delivery vector caused insertional activation of a gene and, 27 months after gene therapy, one subject died from sepsis while the other has undergone successful allogeneic HSC transplantation[205]. Haemophilia is an inherited bleeding disorder caused by a deficiency of functional clotting factors VIII or IX in the blood plasma that can be life threatening. Several strategies have been considered for the development of haemophilia gene therapy, which have resulted in stable correction of the bleeding problem, both in murine and canine models (reviewed in [167]).

Other genetic disorders where gene therapy has been fairly applied with significant advances include congenital blindness and muscular dystrophy, among others. For instance, recombinant AAV vectors carrying the human RPE65 gene have been shown to restore vision in animal models that resemble RPE65-associated retinal disease called Leber congenital amaurosis, an incurable retinal degeneration, which causes severe vision loss. A clinical trial to assess the safety of rAAV2-hRPE65 in subjects with the disease reported increased visual sensitivity without vector-related serious adverse events detected or systemic toxicity[82]. The safety and efficacy of this gene transfer was extended to at least 1-year post treatment[43] and phase III clinical trials are ongoing.

Gene therapy for acquired diseases such as cancer, neurodegenerative diseases (example Parkinson's Disease, Huntington's Disease) and other acquired diseases such

as viral infections (e.g. influenza, HIV, hepatitis), heart disease and diabetes have also been performed and advanced to clinical studies.

A large part of all clinical trials are gene therapy trials for cancer and many of these are entering the advanced stage, including a phase III trial of Ad.p53 for head and neck cancer (reviewed in[155]) and phase III gene vaccine trials for prostate cancer[133]. Among the various gene therapy strategies that have been employed to treat many types of cancer are suicide gene therapy, oncolytic virotherapy, anti-angiogenesis and therapeutic gene vaccines. For instance, a phase II study of the efficacy, safety and immunogenicity of OncoVEXGM-CSF, a replication-competent HSV-1 vector, in patients with different stages of malignant melanoma is underway (ClinicalTrials.gov NCT00289016). There is a phase I study for the treatment of recurrent ovarian cancer (ClinicalTrials.gov NCT00964756) based on a preclinical suicide gene therapy safety study using a tropism modified bicistronic adenovirus (Ad5.SSTR/TK.RGD) with capability for non-invasive imaging of gene transfer in patients[135].

Searching the clinical trials database mentioned above, one can find many clinical studies for HIV infection treatment, consisting mainly in the evaluation of the safety and immune responses to a possible vaccine. For example, one of the most recent phase I clinical trial was a study of MVA-CMDR, a candidate HIV-1 vaccine based on a recombinant modified vaccinia Ankara viral vector expressing HIV-1 genes env/gag/pol. This study demonstrated that vector administration was safe, well-tolerated and elicited durable cell-mediated and humoral immune responses[50]. ClinicalTrials.gov identifier number NCT00376090.

1.7 Human T-cell acute lymphoblastic leukaemia (T-ALL): the model for our delivery and targeting strategy

ALL is the most common type of leukaemia and is a rapidly progressing disease. In this study, to investigate the ability of the Sindbis/anti-FITC pseudotyped lentiviral vector to mediate targeted cell transduction and gene delivery *in vivo* it was used a T-ALL mouse model, in which leukaemia is induced by transplantation of the T-ALL cells.

1.7.1 Overview of the disease

ALL most likely originates from various genetic lesions in blood-progenitor cells that are committed to differentiate in the T-cell or B-cell pathway, including mutations that give the capacity for unlimited self-renewal and those that lead to precise stage-specific developmental arrest [176]. Leukaemia cells in ALL have clonal rearrangements in their immunoglobulin or T-cell receptor genes and express anti-receptor molecules and other differentiation-linked cell-surface glycoproteins that basically run through those of immature lymphoid progenitor cells within the early stages of normal T and B lymphocytes development[176]. T -cell acute lymphoblastic leukaemia is a neoplastic disease of the T-lymphocytes that is linked with a poor prognosis. It represents 15% of childhood and 25% of adult ALL[176]. Compared with the more common B-cell-lineage ALL, T-ALL is defined by distinct clinical and biological characteristics and is generally associated with more unfavourable clinical features, such as a high whiteblood-cell count, bulky adenopathy and involvement of the central nervous system[223]. However, despite these features, the outcome for patients with T-ALL has improved markedly in recent years owing to the application of intensive chemotherapy regimens but even though, significant short-term and potentially long-term side effects occurs.

Although current treatment protocols have improved the overall outcome for patients with T-ALL, a significant number of patients remain at a high risk of relapse with severe complications resulting from the intensive regimens, and few individuals survive when the disease recurs.

T-ALL is considered to result from the malignant transformation of normal developing T cells in the thymus, the thymocytes. Signalling pathways that control T-cell development in the thymus or are involved in T-cell activation are important in its development. Therefore, deregulated signalling is considered a major contributing factor in leukemogenesis of T-ALL.

Additionally, oncogenic transcription factors are expressed aberrantly in leukaemia cells. Just to give an example, MYB oncogene is frequently duplicated in human T cell acute lymphoblastic leukaemia (T-ALL)[110]. Cytogenic analyses in lymphoblasts reveal that there are a good percentage with chromosomal translocations that activate a small number of oncogenes and deletions that lead to losses of tumour suppressor genes. Indeed, chromosomal translocations are a hallmark of ALL; however they must act in concert with several other genetic lesions to induce an evident leukaemia. In T-ALL at least five multi-step mutational pathways leading to leukaemia have been identified, and in some cases these pathways involve several genetic lesions[51].

Four major classes of mutations are involved in the molecular pathogenesis of T-ALL: cell cycle defects (96% attributed to mutations in CDKN2A/2B); differentiation impairment (with the highest percentage given to mutations in TAL1 plus LMO1/2 genes); most mutations implicated in proliferation and survival are still to be identified, but ABL1-fusions can account for 8%; and finally, more than fifty percent of mutations concerning self-renewal capacity comprise NOTCH-1, with the remaining still unknown[51]. NOTCH-1 is a gene codifying for a transmembrane receptor that regulates normal T-cell development. The exact mechanisms by which alterations in NOTCH-1 signalling cause T-ALL are still unclear but probably is related to constitutive expression of oncogenes such as MYC and cooperation with other signalling pathways[177].

1.7.2 Treatment and new therapies

Current treatment is based on conventional chemotherapy and HSCT that has developed to act in conjunction with the former or as replacement with the aim of improving survival and quality of life.

Targeting many of the molecules involved in the pathogenesis of T-ALL can be used as a therapeutic strategy for the treatment of the disease. Since NOTCH-1 activation is known to play a crucial role in T-ALL pathogenesis[233], pharmacologic inhibition of NOTCH-1 by gamma secretase inhibitors (GSI) is a therapeutic strategy in T-ALL[116, 212]. However, only a few T-ALL cell lines show sensitivity in terms of the growth inhibitory effect. In a subsequent study, small-molecule inhibitors, including heat-shock protein 90, histone deacetylase, PI3K/AKT, and proteasome inhibitors, could reverse the gene expression changes induced by NOTCH1. Furthermore, most of the inhibitors synergized with GSI *in vitro* in suppressing T-ALL cell growth in GSI-sensitive cells[182].

More recently, therapeutic antibodies were generated to target specifically each of the NOTCH receptors alone with the advantage of reducing the toxicity associated with inhibition of both NOTCH 1 and 2 by those gamma secretase inhibitors[235].

Additionally, the immunotoxin HA22 (containing an Fv fragment anti-CD22 fused to truncated Pseudomonas exotoxin), which is an improved version of the mentioned above BL22 with higher affinity to CD22, is undergoing clinical testing in HCL, CLL, non-Hodgkin's lymphoma, and also paediatric ALL (reviewed by[107]).

The progress in understanding the biology of the disease and the underlying molecular mechanisms will contribute to the design of new alternative or complementary therapies.

2. Materials and methods

2.1 Cell lines

HeLa cells, 293T cells and 293DTR cells[119] (kindly provided by Dr. Ronald Rodriguez) were grown in Dulbecco's modified Eagle medium (DMEM - Lonza) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine and 100 U/ml penicillin, streptomycin and amphotericin (Lonza). Jurkat (E6-1 clone, ATCC) and Molt-4 (ATCC) cells were cultured in RPMI medium (Lonza) supplemented as mentioned above. Cells were maintained up to a month in culture in a humidified incubator at 37ºC and 5% CO_2. Jurkat/GFP-Fluc were obtained by fluorescence activated cell sorting (FACS) of VSV-G/GFP-Fluc transduced cells. Jurkat/DsRed and Jurkat/DsRedIRESRenilla were obtained by transduction of Jurkat cells with VSV-G/DsRed and VSV-G/DsRedIRESRenilla, respectively, followed only by Ficoll density centrifugation to remove dead cells.

2.2 Plasmid construction and Sindbis viral envelope mutation

A recombinant anti-FITC specific single-chain antibody fragment was kindly provided by Dr. K. Dane Wittrup (MIT, Boston, USA)[141]. The 4M5.3 anti-FITC scFv contains 14 mutations from the wild-type scFv and has a 1800-fold increase in fluorescein-binding affinity. A DNA fragment encoding the 4M5.3 scFv modified at 5' and 3' ends with primers to introduce the *Bst*EII cloning sites was generated by polymerase chain reaction (PCR) amplification. The resulting PCR fragment was gel-purified, digested with the restriction endonuclease *BstE*II, and cloned into the *BstE*II restriction sites of the Sindbis envelope expression vector pIntron Sindbis-ST6LL[2] generating the plasmid pIntron Sindbis/anti-FITC[1]. This plasmid has an HA tag in fusion with the scFv. Mutations in the Sindbis envelope, which were already described[152, 162], were generated by the QuikChange II XL site-directed mutagenesis kit from Stratagene using the primers listed in table XI, Appendix A. These mutants were designated as M1, M2, M3 and M4 for simplicity and correspond to: M1: deletion of E3 aa 61-64, M2: E2 SLKQ68-71AAAA, M3: E2 KE159-160AA, M4: E1 AK226-227SG. Therefore, through combination of these mutations it was produced the final plasmids pIntron Sindbis-FITC M123and pIntron Sindbis/anti-FITC M1234. The mutations

[1] This construct was generated by Frederico Aires da Silva.

were confirmed by DNA sequencing and the expression of scFv on the Sindbis envelope was confirmed by western blot with anti-HA HRP antibody (Roche).

The DsRed-Vpr fusion plasmid was constructed by PCR amplification of the DsRed from pIRES2 DsRed-Express2 vector (Clontech) and the Vpr from pEGFP-Vpr (NIH AIDS Research & Reference Reagent program) followed by overlapping. This fusion was then cloned into a vector using the pcDNA™3.1/V5-His TOPO® TA Expression Kit (Invitrogen).

FUW lentiviral vector was obtained by removing the GFP reporter gene from the vector FUGW[127] (http://www.addgene.org/14883/) using the restriction enzymes *Bam*HI/*Eco*RI and blunted by T4 DNA polymerase (Fermentas) and re-ligated by T4 DNA ligase (New England Biolabs).

FUW/DsRed was constructed by PCR amplification of the DsRed from the pIRES2 DsRed-Express2 vector (Clontech), digested with *Bam*HI/*Eco*RI and cloning into FUGW, previously cut with the same enzymes. FUW/DsRedIRESTK, FUW/DsRedIRESRenilla and FUW/RenillaIRESTK were constructed by overlap PCR using the primers and template indicated in Table XII, Appendix A, and subsequent cloning into FUW. Renilla was amplified from the vector pGL4.70 [hRLuc] (Promega). The original plasmids containing DTA (PSA-DTA)[166] and HSV-TK (TK.007)[175] were a kind gift of Dr. Janet Sawicki and Dr. Boris Fehse, respectively. DTA was cloned into FUW by digesting the PSA-DTA plasmid with AgeI and SalI to generate a 1.3 Kb fragment containing the DT-A sequence and blunt ligated to the vector. FUW-Luc was constructed by PCR amplification of the firefly luciferase gene from the pGL3-Basic vector (Promega), digested with *Bam*HI/*Eco*RI and cloning into FUGW, previously cut with the same enzymes.

Dr. Luigi Naldini provided the #318/GFP-FLuc lentiviral plasmid. #318/RFP was generated by AgeI/SalI digestion of an RFP plasmid, to excise the insert, and #318/GFP-Fluc, to excise the vector, and ligated by T4 DNA ligase. This RFP was

further cloned into FUW (FUW/RFP) using the primers listed in Table XII, Appendix A.

The 3rd generation lentiviral vector packaging plasmids pMDL (containing Gag/pol) and pRSV/Rev were described before[60]. The VSV-G envelope plasmid, pMD2.G (http://www.addgene.org/12259/), and the 2nd generation packaging construct pCMVΔ8.9, which carries *gag*, *pol*, *tat* and *rev* genes, are available from Addgene.

For all cloning procedures, ligations and transformations were performed using T4 DNA ligase (New England Biolabs) and *E. coli* XL10-Gold competent cells (Stratagene), respectively, according to suppliers' instructions. Plasmids were prepared using the Genomed midi-prep procedure (Genomed) or using the maxi-prep kit (Roche) in the case of the *in vivo* work. Verification of reporter gene expression for each construct was done after transfection of 293T cells and performing the adequate assays. For each transfection approximately 1×10^5 cells were seeded *per* well of a 24-well plate. Transfections were performed using the TransIT®LT1 transfection reagent (Mirus Bio), according to the manufacturer's instructions, with a ratio of DNA:reagent of 1:3. Assays were performed 48 or 65 h post-transfection.

2.3 Luciferase assay

Cells were seeded in a 24-well plate at a confluence of 1×10^5 cells per well and transfected the following day with the TransIT-LT1 reagent. The total amount of DNA (0.9 µg) was kept constant by addition of an empty plasmid. Approximately 65 hrs later, cells were washed once in phosphate buffered saline (PBS) and lysed in 125 µl potassium phosphate buffer (0.1M potassium phosphate pH 7.8, 1%Triton X-100, 1 mM DTT, 2 mM EDTA) and 20 µl of lysate was incubated with 100 µl of the substrates, according to the Dual-Luciferase® Reporter Assay System from Promega. Luminescence was measured in the Infinite 200 reader (TECAN).

2.4 AlamarBlue® cell viability assay

For the alamarblue assay, 10 µl of the reagent (Invitrogen) was added to 100 µl of cells harvested from a 24-well confluent plate and incubated at 37ºC for 1 or 2 hrs.

Fluorescence was read on the Infinite 200 reader in a white 96-well plate and at 550/600 nm.

2.5 DsRed fluorescence analysis

For red fluorescence quantification, cells were washed once in PBS and 100 µl of PBS-ressuspended cells were applied in a white 96-well plate for fluorescence reading at 554/591 nm on the Infinite 200 reader.

2.6 Viral vector production

When performing the optimization of viral vector production, it was used Lipofectamine reagent (Invitrogen) and Fugene 6 reagent (Roche) at a ratio (DNA:reagent) of 1:2 and 1:3, respectively and according to the manufacturer's instructions. For the calcium phosphate protocol, the following solutions were used: 2.5 M $CaCl_2$, 2×HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) pH 7.05 and 1/10 TE pH 7.6. Total amount of DNA was 5 µg for 6-well plates or 2 µg for 24-well plates. Medium was replaced 2 hrs before transfection and the 2×HBS solution plus the $CaCl_2$/DNA mixture (mixed while vortexing) were incubated for 30-40 minutes before drop-wise addiction to the medium.

The following protocol was the one adopted after the optimization mentioned in the results. For the *in vitro* transduction assays, 293T cells were seeded in 6-well plates at the density of 5×10^5 cells /well (~ 60% confluent), 24 hrs before transfection. Cells were co-transfected with the packaging plasmids Gag/pol (1 µg) and Rev (0.32 µg), the lentiviral vector (1.4 µg) and the envelope plasmid Sindbis/anti-FITC (0.7 µg) or the VSV-G (0.12 µg) using the TransIT transfection reagent mentioned above (ratio DNA:reagent of 1:4). Approximately 65 hrs post-transfection viral supernatants were harvested, filtered through a 0.45-µm filter and concentrated by ultracentrifugation at 50 000 rpm in a TLA-100.3 rotor (Beckman). Each pellet was resuspended in 75 µL cold PBS. Only the Sindbis/anti-FITC pseudotyped lentiviruses were concentrated. AMICON ultra 100K centrifugal filter devices (Millipore) were also tested for viral concentration.

DsRed-Vpr-labelled lentiviral vectors were produced by co-transfection with the plasmid DsRed-Vpr (0.9 μg) in addition to the plasmids used to produce the lentiviruses. The lentiviral transfer vector used was FUW, which does not contain any reporter.

To produce lentiviral vectors expressing the DT-A toxin, 293DTR cells were used instead of 293T.

For the initial *in vivo* assays, cells were seeded in 100 mm plates and transfected with 6× more plasmids as mentioned above and using TransIT transfection reagent. For most of the *in vivo* transduction assays, 293T cells were seeded in 150 mm plates at the density of 9×10^6 cells /plate (~ 75% confluent), 24 hrs before transfection. Medium was changed 2 hrs before transfection. Cells were co-transfected with the packaging plasmids Gag/pol (12.5 μg) and Rev (6.25 μg), the lentiviral vector (25 μg) and the envelope plasmid Sindbis/anti-FITC (9 μg) using the calcium phosphate method by Dr Luigi Naldini's lab. The solutions used were as follows: 2.5 M $CaCl_2$, 2×HBS (100 mM HEPES, 281 mM NaCl, 1.5 mM Na_2HPO_4) pH 7.12 and 1/10 TE pH 8. The 2×HBS solution was added drop wise to the DNA-TE-CaCl2 mixture while vortexing at full speed and the precipitate was added to 293T cells immediately. Between 14-16 hours after transfection, medium was replaced. Forty-eight hours post-transfection, viral supernatant was harvested and filtered through a 0.45-μm filter. The lentiviral vectors were concentrated overnight using a Lenti-X concentrator (Clontech) according to the manufacturer's instructions, followed by an ultracentrifugation using the same conditions as above. Viruses were titrated by measuring the p24 levels with an enzyme-linked immunosorbent assay (ELISA) HIV p24 kit, using either the Innotest HIV antigen mab (Innogenetics) or the HIV-1 p24 antigen capture assay kit (AIDS & Cancer Virus Program, National Cancer Institute at Frederick).

2.7 Western blot and antibodies

Concentrated viral supernatants were subjected to protein denaturation at 100ºC for 5 minutes in 2×SDS-gel loading buffer (2% SDS, 30 mM Tris.HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.05% bromophenol blue). Samples were loaded on a

10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Scheiller & Schuller). After staining with Ponceau S (Sigma) to verify the uniformity of protein load and transfer, the membrane was incubated for 1 h at room temperature in blocking buffer (Tris buffered saline pH 7.4 containing 0.05% Tween-20 (TBST) and 5% skim milk) and immunoblotted with the respective antibodies. Membranes were washed 6 × 10 minutes in TBST after incubation at room temperature with the primary (1 hr[1]) and secondary (30 min.) antibodies. Antibodies were diluted in the blocking solution. Anti-HA horseradish peroxidase (HRP)-conjugated mouse monoclonal antibody (Roche) was diluted 1:1000. Human anti-p24 antibody (NIH AIDS Research & Reference Reagent Program) was used at a dilution of 1:5000. Rabbit polyclonal Sindbis antiserum (diluted 1:2500) was a kind gift of Dr. Peter Bredenbeek. HRP-conjugated secondary antibodies (Bio-Rad) were used at 1:10000 dilution. Proteins were detected by enhanced chemiluminescence SuperSignal (PIERCE).

2.8 *In vitro* binding of Sindbis/anti-FITC envelope expressed in 293T cells

Cells were plated on 24-well plates (1×10^5 *per* well) and transfected the following day with Sindbis/anti-FITC M123 plasmid using the TransIT transfection reagent. Two days later, cells were washed twice with PBS and increasing concentrations of anti-CD7-FITC (BD Biosciences) were added for 30 minutes on ice. After another washing step, cells were ressuspended in PBS and analysed by flow cytometry (BD FACSCalibur) for $FITC^+$ cells.

To check anti-FITC scFv expression at the cells surface, transfected cells were incubated with rat anti-HA HRP (1:1000) for 30 minutes on ice, washed with PBS/1% BSA and incubated with secondary anti-mouse Cy5 (Jackson ImmunoResearch) at 1:2500 dilution for another 30 minutes on ice. Cells were washed one more and analysed by flow cytometry for $Cy5^+$ cells.

[1] 2 hrs in the case of Sindbis antiserum.

2.9 *In vitro* binding of lentiviral vectors

Five μg/mL of anti-CD7-FITC antibody was incubated with 150 ng of Sindbis/anti-FITC M1234 pseudotyped lentiviruses (total volume of 150 μL in PBS) at 4ºC for 1:30 hrs to allow binding. Jurkat cells (5×10^5, seeded the day before) were added and incubated for another 1:30 hrs at 4ºC. Cells were spun down at 450 ×g for 5 minutes at 4ºC and washed twice with PBS and then fixed with 4% paraformaldehyde, for 20 minutes at room temperature (RT). Cells were washed and ressuspended in 500 μL PBS for analysis either by flow cytometry or by immunofluorescence for binding ($DsRed^+$) and labelling ($FITC^+$).

2.10 Immunofluorescence staining

For the immunofluorescence, 293T cells were seeded at a confluence of 4×10^5 cells *per* well in a 6-well plate containing 13 mm ∅ coverslips previously coated with poly-D-lysine (Sigma) and transfected as described before with pIntron Sindbis/anti-FITC. Forty-eight hours after transfection, cells were washed for 5 minutes in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde, for 20 minutes at RT. For Jurkat cells, after fixation, they were cytospun at 800 rpm for 5 minutes. Cells were washed twice in PBS and permeabilized with 0.1% Triton X-100 in PBS, for 20 minutes at RT. After washing with 0.05% Tween 20 in PBS, cells were blocked in this washing solution plus 5% normal goat serum for 1 hr at RT. Cells were incubated for 1 hr at RT with rabbit anti-Sindbis serum diluted 1:500 and goat anti-rabbit IgG (H+L) rhodamine secondary antibody (PIERCE), at 1:500 dilution, for 45 min at RT and in the dark. Cells were always washed 3 times after each incubation with antibody. A drop of DAPI (at a concentration of 50 ng/ml) was used to stain the nucleus. Coverslips were mounted in Fluoromount-G medium (Beckman Coulter) and cells were visualized using an Olympus IX-50 inverted microscope.

2.11 Targeted *in vitro* transduction

Lentiviral vectors expressing DsRed (~200 ng HIV p24) were added to retronectin (Takara Bio)-coated 24-well plates (40-60 μg/mL per well) and centrifuged at 2000xg for two hrs at 32ºC. Jurkat cells (8×10^4, seeded 24 hrs before), FITC-conjugated

antibodies (5 μg/mL) and polybrene (8 μg/mL) were added to the plates with the lentiviruses (800 μl total volume *per* well) and spinoculated for 1:30 hrs at 930×g and 32ºC. Cells were incubated overnight at 37ºC and 5% CO_2. The next morning, medium was removed and cells were treated with a low-pH buffer (0.131 M citric acid, 0.066 M Na_2HPO_4, pH 5), for approximately 1 min at RT. The buffer was replaced with culture medium and the cells were incubated for two more days. Jurkat cells were acquired for DsRed (transduction efficiency) and for FITC (cell labelling) by flow cytometry. Data analysis was performed using the FlowJo software. The anti-CD7-FITC and anti-CD19-FITC antibodies were purchased from BD Biosciences and the FITC anti-HLA class I (clone W6/32) was purchased from Sigma.

2.12 Competition assay

For *in vivo* competition assays, the procedure was the same as for the *in vitro* transduction assays but with the addition of increasing concentrations of mouse IgG1 anti-FITC (clone FIT-22, Biolegends) or isotype control antibody (Biolegends) to the mixture of cells, anti-CD7-FITC and virus, before the spinoculation.

2.13 HSV-TK/GCV suicide gene assay

For GCV titration, DsRedIRESTK transfected 293T cells or VSV/DsRedIRESTK transduced Jurkat cells were treated with several concentrations of GCV for a period of 5 days, starting on the day following transfection or transduction, respectively. Jurkat cells were transduced with Sindbis/anti-FITC M1234 lentiviruses expressing DsRedIRESTK as mentioned before and after acidic buffer treatment on the next day, a new medium was added containing 1 μg/mL of GCV. Cells were removed at several time points for flow cytometry analysis ($DsRed^+$ cells) and to the remaining cells it was added more GCV supplemented medium.

2.14 Mice

Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were obtained from Charles River Laboratories, Rag2-/-γc-/- were obtained from IMM, Balb/c were from IMM and NOD.Cg-Rag1IL2rg (abbreviated as NOD-$Rag1^{-/-}$IL2r$\gamma^{-/-}$)

were obtained from The Jackson Laboratory. Mice were housed at IMM animal facility in pathogen-free conditions. At the first indication of morbidity (weight loss, lethargy, ruffled fur), or no more than 5 weeks following cell inoculation, mice were killed by cervical dislocation.

2.15 In vivo targeted cell transduction

Twenty million Jurkat/GFP-Fluc cells were injected through the tail vein of female NOD-Rag1^{null} Il2rγ^{null} mice, which were irradiated the day before using a 137Cs source (Gammacell ELAN 3000 irradiator) at a dose of 550 cGy [164] to induce leukaemia. D-luciferin (3 mg /mice in DPBS – Caliper Life Sciences) was administered intraperitonially[1] 1 week after cell injection to check for engraftment under a CCD (charged-coupled device) camera (IVIS Lumina - Xenogen). Mice were anesthetized before imaging with an i.p. injection of ketamine/xylazine. The following day (or 3 days later), Sindbis/anti-FITC pseudotyped lentiviruses expressing RenillaIRESDsRed (or RenillaIRESTK) (~3µg of HIV p24) were injected through the tail vein and its biodistribution was analysed 4 days later by intravenous administration (retro-orbital injection) of 100µg of coelenterazine[2] (benzyl-coelenterazine (h-CTZ) in sterile injection vials, NanoLight Technology) *per* mice. Acquisition time for both luciferases was 3 min, bin large, f/stop 1. At the 3rd or 4th week after cell injection, mice were killed and the femurs and spleens were removed. Bone marrow and spleen cells, collected in PBS, and blood cells (lysed with an eBioscience lysis buffer) were subjected to flow cytometry analysis to evaluate the percentage of engraftment (GFP^+) and transduction (GFP^+DsRed^+). The CD45-FITC antibody used to stain human engrafted cell was from eBioscience. Coelenterazine stock solution was prepared at 5 µg/µl in provided diluent. Working solution was diluted in PBS, containing only 5 mM NaCl, pH 7.2 [213]. Each mouse received 150 µl of this coelenterazine solution.

2.16 Real-time PCR

Real-time PCR was performed to quantify the vector copy number in the genomic DNA isolated from mice. Bone marrow was collected by flushing the femurs with PBS

[1] Acquisition was started 10 minutes after injection.
[2] Acquisition was started 1 minute after injection.

and genomic DNA was isolated using the innuPREP DNA Mini Kit (Analytic Jena Life Science), according to the supplier's instructions. Quantification of vector copy number and cell number was performed by using SYBR Green (Fermentas) and an ABI PRISM 7300 sequence detector (Applied Biosystems). All reactions were performed with 0.5 ng of DNA, 12.5µl of SYBR Green PCR master mix and 7.5 pmol forward and reverse primers in a final reaction volume of 25 µl. The primers for analysis of vector copy number (Renilla luciferase) are indicated in Table XIII, Appendix A. The standard for quantification of vector copy number was FUWDsRedIRESRenilla. Quantification of the cell number was performed using primers for human β-actin (Table XIII, Appendix A) and the standard was made using genomic DNA isolated from known numbers of Jurkat cells.

3. Results and discussion

3.1 *In vitro* gene therapy

3.1.1 Generation of mutations on the Sindbis glycoproteins and expression of anti-FITC scFv at the surface of Sindbis-pseudotyped lentiviral vector

A recombinant anti-FITC specific scFv was cloned between amino acids 71 and 74 of the E2 Sindbis glycoprotein by substitution for ST6 scFv in the envelope expression vector pIntron Sindbis-ST6LL[2]. The chimeric Sindbis envelope encode the 4M5.3 scFv in which the N-terminal VL region is linked with the VH region through the 18-aa peptide linker[141]. This chimeric Sindbis/anti-FITC envelope has also an HA tag at the C-terminus of the scFv to facilitate its detection. To evaluate the successful incorporation into the lentiviral vector and the expression of the anti-FITC at the surface, viral particles were produced by transfection of 293T cells and the virus-containing supernatant was collected approximately 68 hours post-transfection, concentrated by ultracentrifugation and ressuspended in 2×SDS loading buffer. Expression of the anti-FITC at the surface of the virus was confirmed by Western Blot using both an anti-Sindbis serum and an anti-HA HRP antibody (Figure 4), which detected a band of approximately 80 KDa, corresponding to the chimeric Sindbis envelope.

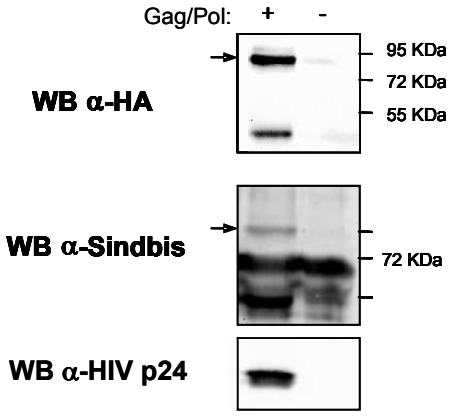


Figure 4. Detection of Sindbis/anti-FITC envelope on pseudotyped virions. Viral supernatant harvested from transfected 293T cells was concentrated by ultracentrifugation and the pellet was ressuspended in 30 µl 2×SDS-loading buffer and applied on a 10% SDS-PAGE. Western blot with an anti-HA HRP antibody reveals a band of approximately 80 KDa corresponding to the Sindbis envelope. The same band is detected with an anti-Sindbis virus serum. Supernatant harvested from cells where gag/pol plasmid was absent was included as a negative control.

Clinically effective gene therapy protocols for various diseases would ideally use procedures for efficient and specific targeting of therapeutic genes to the affected cells while maintaining stable transduction and long-term expression. This can be accomplished by direct injection into the bloodstream followed by homing of the vector to the desired target cells or organs. For the success of this strategy with chimeric Sindbis/anti-FITC envelope, the background non-specific infection of wild type Sindbis envelope has to be reduced. Sindbis virus envelope is able to pseudotype oncoretroviruses and lentiviruses and it has an entry mechanism via low-pH mediated endocytosis. It is commonly used due to its high levels of expression and production of high-titer infectious particles. Even though effective *in vitro*, it has a nonspecific cell tropism *in vivo* as intravenous injection of Sindbis envelope pseudotypes into mice results in higher levels of infectivity in liver and spleen cells. This occurs because its receptors, the high-affinity laminin and heparin sulphate, have wide distribution and are highly conserved[148]. It has been shown that the display of exogenous protein domains or scFv can target lentiviral vectors to specific cells[147, 2, 152] and with the further addition of mutations in the envelope glycoprotein it would eliminate the problem of its non-specificity. Moreover, the insertion of the scFv at that position (E2 aa 71) will also eliminate Sindbis original tropism. Sindbis virus has three envelope glycoproteins: E1, E2 and E3. E2 binds to the host cell receptor, E1 mediates membrane fusion in a low pH-dependent fashion and E3 works as a signal sequence peptide for E2 protein. Therefore, some domains of the these Sindbis envelope proteins were mutated using the Quickchange II XL Site Directed mutagenesis kit to reduce inherent non-specific infection. The list of oligonucleotides used for each mutation can be found in table XI, Appendix A. These mutations have been previously described[152] and will be defined here as M1, M2 and M3, which corresponds to (Figure 5): M1: deletion of E3 aa 61-64, resulting in higher selectivity, but decreased virus titer; M2: E2 SLKQ68-71AAAA that enhances titer without altering specificity; M3: E2 KE159-160AA, which enhances selectivity.

The final chimeric Sindbis envelope will have a combination of these mutations. Nevertheless, Pariente and colleagues have additionally modified the Sindbis virus envelope containing the three mutations described above (M123) by introducing the AK226-227SG mutation in the E1 glycoprotein [162]. These mutations have been

described to mediate fusion in the absence of cholesterol in the target membrane[129], increasing the tropism and the infectivity of Sindbis pseudotyped vectors[150]. Therefore, it was created a modified Sindbis envelope with these four mutations combined to use in the *in vivo* studies in order to reduce endogenous Sindbis virus tropism. These mutations were confirmed by sequencing and the expression of the anti-FITC at the surface of the virus, called Sindbis M1234 for simplification, was confirmed by Western Blot with an anti-HA HRP antibody.

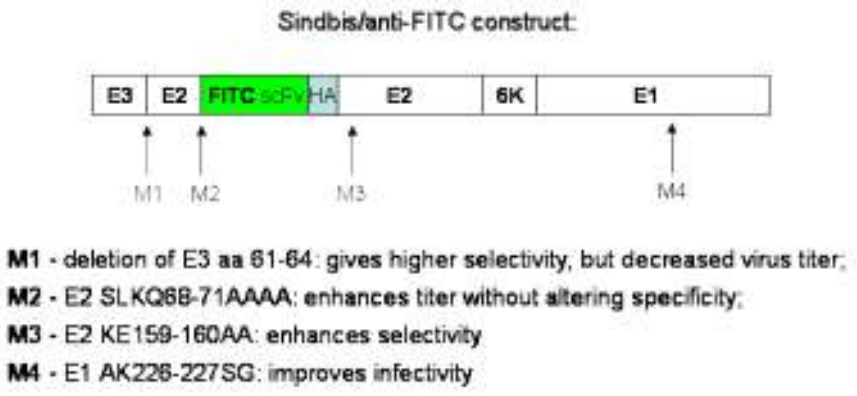


Figure 5. Schematic of Sindbis/anti-FITC construct with the several mutations depicted by an arrow.

One disadvantage of the noncovalent conjugation of vectors with antibodies reported by Morizono and colleagues[147, 152] is that in immunocompetent animals, serum immunoglobulin will compete with conjugated antibodies for biding to the ZZ domain of the envelope protein. In the strategy herein proposed this problem does not arise since FITC is a small organic molecule not present in the serum and therefore, it avoids competition between scFv anti-FITC and the immunoglobulins present in the serum. Another favourable feature of this scFv is the fact that it is an improved version with enhanced fluorescence and increased binding and therefore, the titers of the pseudotyped vector (and consequently the transduction efficiency) will be also improved as that is dependent on the affinity and specificity of the inserted scFv.

With the insertion of the scFv on the Sindbis envelope and the created mutations, the binding of the pseudotyped lentiviral vector will depend only on the affinity of the scFv for the FITC on the surface of labelled cells.

Probably multiple insertions of the scFv into the envelope proteins would increase the avidity of the pseudotyped vector for target cells and consequently the viral titer, but one could not predict the effect it would have on the folding and assembly of the entire envelope and consequently, on its fusion ability.

3.1.2 Expression of the Sindbis envelope at the surface of 293T cells

In order to check the display of the FITC scFv in the virus producing cells and evaluate the amount of the Sindbis envelope plasmid that should be used for transfection to allow the highest expression possible at the surface of 293T cells, the cells were transfected with three different amounts of Sindbis/anti-FITC M123 envelope. Forty-eight hours later, cells were stained with an anti-HA HRP as primary antibody and with anti-mouse Cy5 as secondary and analysed by flow cytometry (Figure 6). The percentage of Cy5 positive cells indicates the amount of the envelope protein displayed at the surface of 293T cells. This result indicates that the best display is achieved using 0.5 μg of the plasmid envelope.

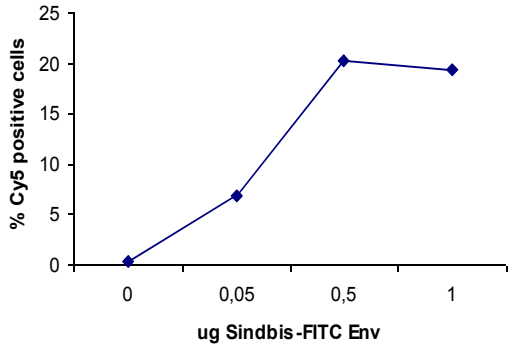


Figure 6. Sindbis/anti-FITC M123 expression at the surface of 293T cells. Cells were transfected with the indicated amounts of Sindbis/anti-FITC M123 envelope. Forty-eight hours later, cells were collected, stained with an anti-HA HRP antibody followed by an anti-mouse Cy5 and analysed by flow cytometry. Cy5 positive cells indicate the amount of envelope displayed at the surface of 293T cells. This is a representative of two independent assays.

To further demonstrate the surface expression of Sindbis/anti-FITC on 293T cells, it was used an indirect immunofluorescence assay with rabbit anti-Sindbis polyclonal serum as primary antibody and anti-rabbit rhodamine as the secondary antibody. In Figure 7, despite not including a nuclear staining, one can easily observe the cell membrane stained in red, clearly showing the expression of Sindbis envelope at the surface of 293T cells transfected with the plasmid Sindbis/anti-FITC M1234.

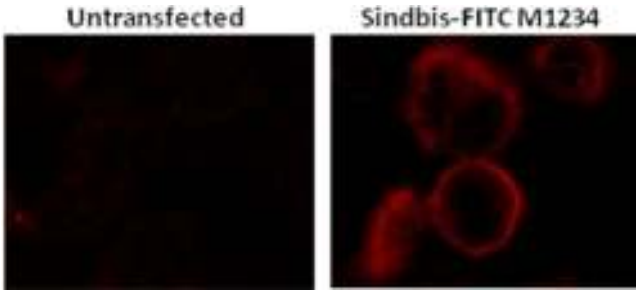


Figure 7. Sindbis/anti-FITC M1234 expression at the surface of 293T cells. The cells were transfected with Sindbis/anti-FITC plasmid and 48 hours later an immunofluorescence assay was performed using anti-Sindbis polyclonal serum as primary antibody and an anti-rabbit rhodamine as secondary antibody, both at 1:500 dilution, and slides were visualized under the microscope.

3.1.3 Binding of the Sindbis/anti-FITC M123 envelope to the anti-CD7-FITC tested in 293T cells

The antibody chosen for T-cell labelling in our system was the FITC-conjugated CD7 antibody because CD7 is a surface antigen abundantly present on the majority of T cells and is rapidly internalized after antibody binding. The capacity of Sindbis/anti-FITC M123 envelope for binding to the anti-CD7-FITC was tested in 293T cells. Therefore, increasing amounts of the antibody were incubated with cells expressing the three different quantities of envelope tested above, 0.05, 0.5 and 1 μg (Figure 8). Analysis of the FITC expression by flow cytometry indicated the percentage of binding. One can notice that the augment in binding, which is exclusively mediated by the scFv as CD7 receptor is T cell-specific and is not present in 293T, is proportional to the amount of CD7-FITC antibody and somewhat stabilizes from 10 μg/mL, most likely because all binding sites were already occupied. Consequently, this assay allowed us to determine that the concentration of FITC-conjugated antibody from which we can obtain a good cell labelling is 2.5 μg/mL and confirmed as well that the amount of envelope for the best display of anti-FITC is 0.5 μg.

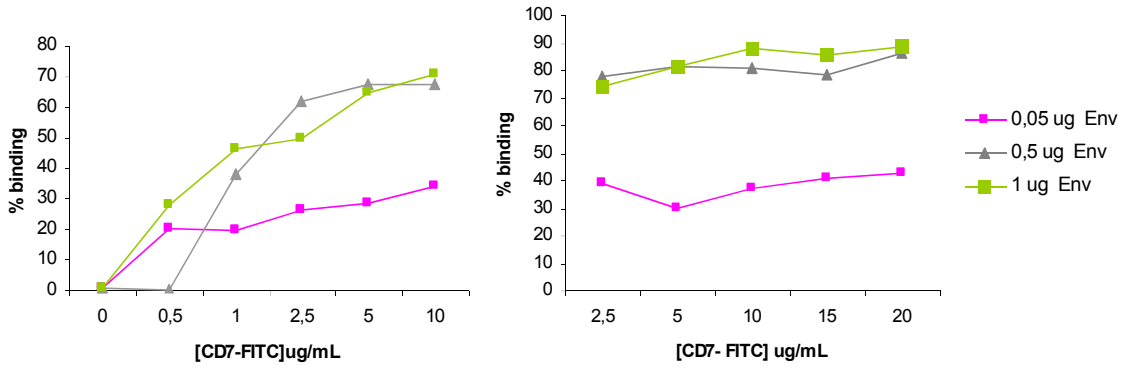


Figure 8. Binding of Sindbis/anti-FITC M123 to the CD7-FITC antibody tested in 293T cells. Titration of the anti-CD7-FITC antibody for binding to the different amounts of Sindbis/anti-FITC M123 envelope tested, in two separate assays. Cells were transfected with 0.05, 0.5 or 1 μg of envelope plasmid and 48 hrs later incubated at 4ºC with increased concentrations of the anti-CD7-FITC antibody. After washing, cells were analysed for binding (FITC positive) by flow cytometry. This is a representative of two independent assays.

3.1.4 In-vitro binding of Sindbis/anti-FITC pseudotyped lentiviruses to Jurkat/CD7-FITC cells

Prior to starting the transducing of the target cells, it was important to demonstrate binding of the Sindbis-pseudotyped lentiviruses to the cell membrane. Given that the anti-mouse Cy5 secondary antibody previously used in the staining of the Sindbis/anti-FITC envelope at the surface of 293T cells will also recognize the mouse anti-human CD7-FITC besides the rat anti-HA HRP (used as primary for staining the Sindbis/anti-FITC envelope), it was necessary to use another method for detecting virus binding at the cell surface. It was shown that GFP-vpr can be incorporated into the virion when the GFP-vpr plasmid is supplied *in trans* during viral preparation[138]. The resulting virus is labelled by GFP and could be detected by green fluorescence. Because the antibody used for labelling is FITC-conjugated (CD7-FITC), the lentivirus would have to be labelled with a different colour. Therefore, it was constructed a plasmid that expresses DsRed fused to the N-terminus of HIV-1 vpr (designated DsRed-vpr). However, the expression of the DsRed from this plasmid in 293T cells is much more reduced as compared to the lentiviral plasmid FUW/DsRed (14% *versus* 62%, respectively, as analysed by flow cytometry). Nevertheless, viruses were produced with the backbone plasmid FUW that lacks the transgene and co-transfection of the plasmid expressing DsRed-Vpr. Different amounts of lentiviruses were incubated with Jurkat cells plus anti-CD7-FITC at 4ºC, to avoid fusion. Binding was analysed by flow cytometry but no red fluorescence was detected, despite the good cell surface labelling with CD7-FITC antibody (Figure 9).

Several studies by Pin Wang's group have used the GFP-vpr labelling scheme[89, 245, 113, 211] either to demonstrate the presence of the Sindbis envelope at the surface of vector particles by co-localization of the vector core and the stained envelope, by imaging only the lentiviral vectors, or to demonstrate lentiviral vector binding to cells. Curiously, they employ the GFP-vpr labelling scheme only with confocal microscopy. When using a flow cytometry approach to detect binding, they only perform the staining of the fusogenic molecule derived from the Sindbis virus glycoprotein via indirect immunofluorescence.

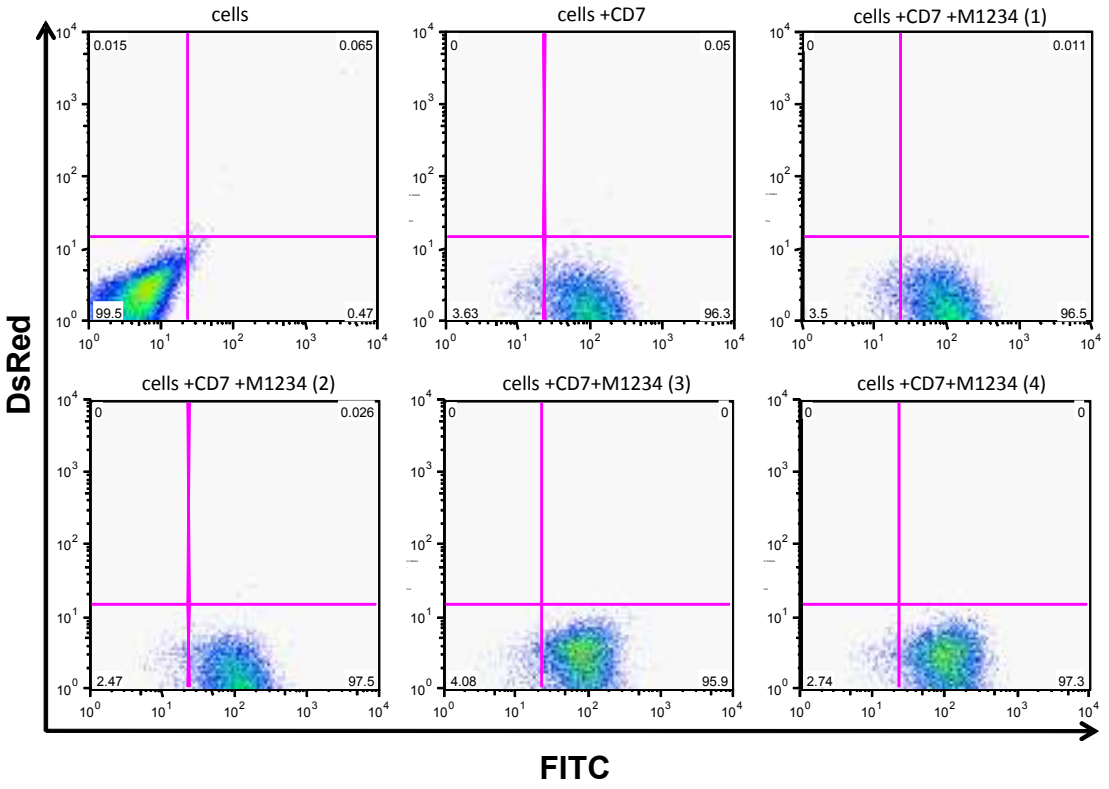


Figure 9. *In vitro* binding assay in Jurkat cells assessed by flow cytometry. Different viral concentrations: (1) 312 ng; (2) 156 ng; (3) 78 ng and (4) 39 ng (HIV p24) of Sindbis/anti-FITC M1234 lentiviruses labelled with DsRed were incubated with anti-CD7-FITC (5µg/mL) for 1:30 hrs on ice. Then, 5×10^5 Jurkat cells were added and incubated for another 1:30 hrs. For viral production 0,3 µg DsRed-Vpr was used in transfection. Cells were analysed by flow cytometry for FITC (surface-labelled cells) and DsRed (cell-binding viruses) fluorescence.

Therefore, we have opted for another technique to demonstrate the binding of the virus to the cell. For that, an indirect immunofluorescence assay was employed, which used a rabbit anti-Sindbis polyclonal serum to stain the viral envelope and anti-rabbit rhodamine as a secondary antibody, given that we were able to show the display of the Sindbis/anti-FITC envelope at the surface of 293T cells before using this approach. The binding procedure was the same as the one mentioned above and the immunofluorescence performed exactly as described for Sindbis/anti-FITC detection on 293T cells. Binding of the virus (in red) to the cell membrane (in green) (Figure 10) is only detected when anti-CD7-FITC is present at the cell surface, while no detectable virus is observed on the surface of Jurkat cells alone.

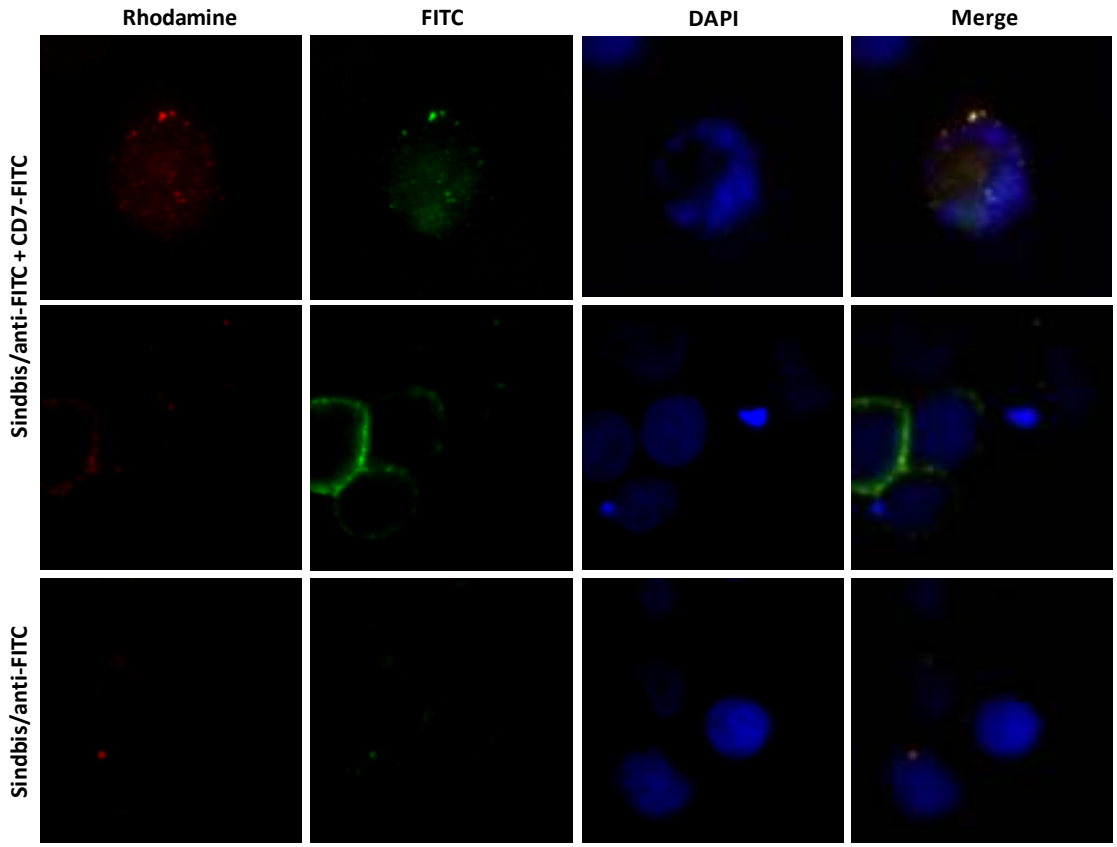


Figure 10. *In vitro* binding assay in Jurkat cells assessed by microscopy. Sindbis M1234/anti-FITC pseudotyped lentiviruses (bearing no reporter gene) were incubated with or without anti-CD7-FITC (5 µg/mL) for 1 hour at 4ºC and after that, cells were added and incubated for another hour. An immunofluorescence assay was performed as mentioned in the Figure 7. Lentiviruses are stained in red, the CD7-FITC antibody in green and the nucleus in blue.

Thus, so far, it was demonstrated that the anti-FITC scFv is exposed on the virion and accessible to the receptors expressed on target cells. The binding specificity of the Sindbis-pseudotyped lentiviral vector is determined by the specificity of the anti-FITC scFv. As mentioned before, the 4M5.3 scFv (anti-FITC) was improved to have a higher fluorescein-binding affinity. It seems that (or we may say that) the fusion of the anti-FITC scFv with the Sindbis envelope proteins did not change its structure what could lead otherwise to loss of their function. Moreover, if the scFv was inserted into the regions of envelope proteins which are difficult to access, the chimeric proteins (scFv plus envelope) would not be able to bind the targeted molecules on the cells.

3.1.5 Production of lentiviral vectors: optimization to reach the highest levels of transduction

For an efficient gene therapy, it is crucial to use a delivery vector that allows high levels of cell transduction and transgene expression. HIV-1-derived lentiviral vectors were the choice for this kind of gene therapy, mainly because of its capacity to stably integrate into the genome of a wide range of cells, both dividing and non-dividing, allowing long-term transgene expression and due to its safety. Several factors may influence lentiviral titer and consequently transduction efficiency. These can be the amount of plasmid transfected into the packaging cells, the type of transfer vector and its internal promoter, the transgene, viral vector collection time and concentration (e.g. ultracentrifugation), the presence of serum in the medium (during vector production and during transduction) and even the type of envelope used to pseudotype the lentiviral vector[125]. Viral infectivity (and titer) is also dependent on the type of transfection reagent used for virus production and the type of cells used for targeting. Therefore, it is very important that all these variables be optimized in order to achieve the best viral titers, as that is critical for an efficient gene delivery.

Available in the lab were the lentiviral packaging plasmids from the 2nd generation, CMVΔ8.9, and the 3rd generation, pMDL (containing Gag/pol) and pRSV/Rev. For pseudotyping, VSV-G was the choice for this initial optimization steps because of its wide tropism and ability to pseudotype a vast range of viral vectors, its stability and high titers of viral production. The lentiviral transfer plasmids used initially in the experiments were the #318/GFP-Fluc and the RFP, but later on these were replaced by FUGW and FUWDsRed (Figure 11). However, to evaluate the transduction efficiency in our Sindbis/anti-FITC system, one would have to use a red fluorescent plasmid as reporter gene due to the overlap between the GFP and the FITC emission spectra.

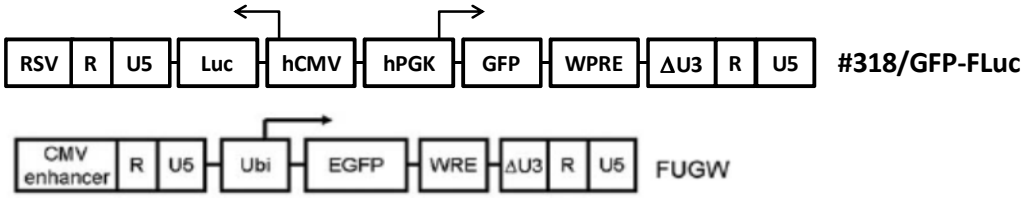


Figure 11. Schematic diagram of the lentiviral transfer vectors FUGW and #318/GFP-Fluc. FUGW and #318/GFP-Fluc have the CMV enhancer and the RCV enhancer/promoter, respectively, substituted for the U3 region of the 5'LTR. ΔU3 denotes a deletion in the U3 region of the 3'LTR that renders the 5'LTR of the integrated provirus transcriptionally inactive. GFP expression is driven by the human ubiquitin promoter (Ubi) in the FUGW plasmid and by the human phosphoglycerate kinase (hPGK) promoter in the #318/GFP-Fluc plasmid. The latter has the human CMV promoter to drive the expression of firefly luciferase (Luc). Both vectors have the Woodchuck hepatitis virus post-transciptional element (WPRE) and the central polypurine tract (cPPT) (this one is not depicted).

An ELISA assay was employed to determine the levels of the HIV capsid protein p24, which gives an estimate of the titer based on the amount of physical particles (pp). Previous reports indicated that 1 pg of p24 Gag protein is equivalent to 1-10 infectious viral particles (TU)[242], thus a vector stock with a 100 ng/ml p24 count contains approximately 10^6/ml infectious viral particles or fewer. That same titer would correspond to 10^9 pp based on the estimation that 1 pg of p24 capsid protein represents around 10^4 pp[65].

As a starting point, different ratios of the plasmids required for viral production were tested to see which one gave the highest value of the HIV capsid protein, p24 (Figure 12). 293T cells were transfected by the calcium phosphate method and viruses were harvested 48 hours later for quantification of HIV p24. The ratio of plasmids that gave the best viral titer was 2:1:1:1 (Gag/pol:VSV:transfer vector:Rev) using the vector expressing GFP, whereas the one expressing RFP was always slightly lower, probably because of its toxicity to the cells.

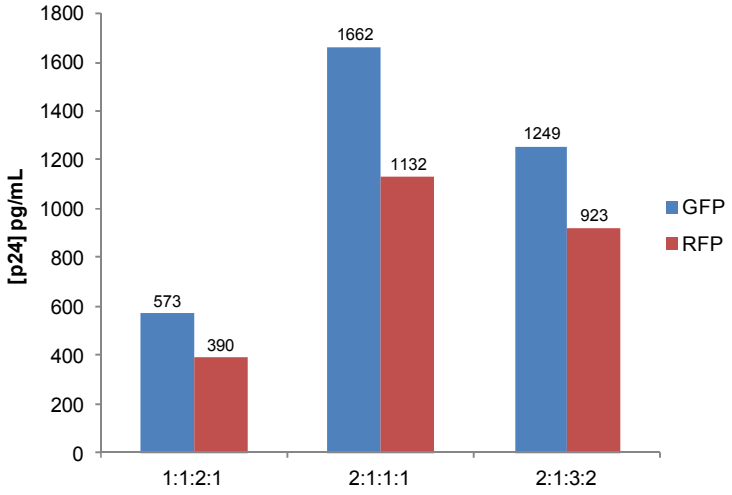


Figure 12. Titration of the plasmid ratio used to produce VSV-G-pseudotyped lentiviruses encoding the transgenes GFP or RFP. 293T cells (5×10^5 *per* well of a 6-well plate) were transfected by $CaPO_4$ precipitation with different ratios of plasmids and each viral supernatant was harvested 48 hrs later for HIV p24 quantification. Depicted in the x axis are the ratios of the Gag/pol:VSV:transfer vector: Rev plasmids.

Sodium butyrate (NaB) is commonly used to increase viral production. It has an effect on transcriptional activity, presumably through up-regulation of viral promoters, enhancing protein production capability[19]. Nevertheless, its addition to the cell culture medium during transfection did not increase much the viral production (Figure 13), although the titers obtained in this experiment were higher than in the previous one. Using the packaging construct CMVΔ8.9, the virus production was lower than using the third generation Gag/pol and Rev constructs with the ratios of 1:2:1:1 and 1:3:1:1 (RFP: Gag/pol: Rev:VSV).

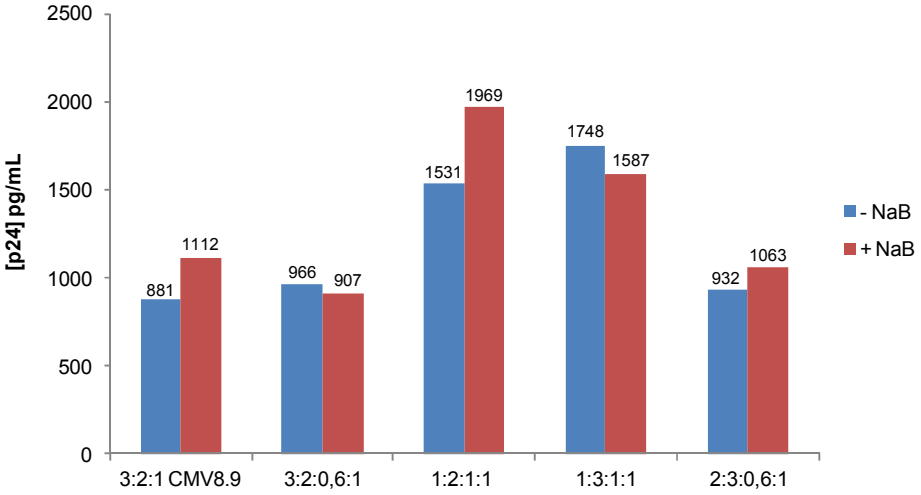


Figure 13. Titration of the plasmid ratio used to produce VSV-G-pseudotyped lentiviruses encoding RFP. 293T cells (5× 10^5 *per* well of a 6-well plate) were transfected by $CaPO_4$ precipitation with different ratios of plasmids (in µg) and in the presence or absence of NaB (4 mM for 22 h). Each viral supernatant was harvested 48 hrs later for HIV p24 quantification. Depicted in the x axis are the ratios of the RFP:Gag/pol:Rev:VSV plasmids.

Next, new viruses were produced either with GFP and RFP vectors, using the ratio of 1:3:1:1 (transfer vector:Gag/pol:Rev:VSV) for cell transfection by calcium phosphate precipitation. The generated lentiviruses were at a concentration of HIV p24 near 7000 pg/ml for GFP and 6000 pg/ml for RFP (Figure 14).

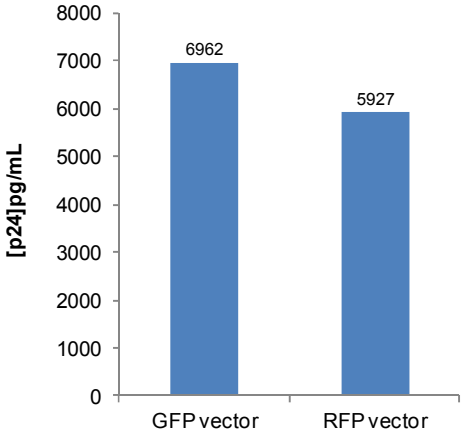


Figure 14. Titers of the VSV-G-pseudotyped lentiviruses encoding GFP or RFP. 293T cells (5× 10^5 *per* well of a 6-well plate) were transfected by $CaPO_4$ precipitation with the plasmid ratios of 1:3:1:1 (Transfer vector:Gag/pol:Rev:VSV). Each viral supernatant was harvested 48 hrs later for HIV p24 quantification.

Both Jurkat and Molt-4 cells were then transduced by spinoculation in 6-well plates for 1 hour at 1200 rpm and 20ºC. The best transduction efficiency was 4% obtained for Jurkat cells infected with the GFP lentiviruses, whereas for Molt-4 the transduction efficiency was only 1.5% (Table II).

Table II. Transduction efficiency of Jurkat and Molt-4 cells infected with the VSV-G-pseudotyped lentiviral vectors expressing GFP or RFP. Cells (8×10^5 *per* well of a 6-well plate) were infected with all the volume of the lentiviruses by spinoculation for 1 hour at 1200 rpm and 20ºC. Three days later, the percentage of transgene expression was analysed by flow cytometry.

CELLS	% FLUORESCENCE		
	Cells only	**GFP vector**	**RFP vector**
Jurkat	0.02	4.00	0.11
Molt-4	0.03	1.48	0.00

Another viral production experiment was set up using two different ratios of plasmids for transfection, either a GFP or a RFP transfer plasmid, and including or not serum in the cell culture medium. Virus were harvested 48 hours post-transfection and concentrated by ultracentrifugation. As it can be observed on Figure 15, the presence of serum in the medium improved viral titers. The results confirms the previous experiments showing that the best ratio of plasmids for transfection is 1:3:1:1 (Transfer vector:Gag/pol:Rev:VSV) but, on the other hand, this time the RFP lentiviral had the highest titer. Nevertheless, this titer was ten times lower than that of the positive control lentivirus included in the ELISA, which was produced using the FUGW lentiviral transfer vector that has the GFP under the control of the UbiC promoter (Figure 11). All the amount of lentiviruses was used to transduce Jurkat cells through spinoculation for 1 hour at 2000 rpm and 32ºC. One can observe that despite the higher titer (and thus slightly higher MOI) of the RFP lentiviruses it did not allowed any cell transduction, opposite to GFP lentiviruses where transduction efficiency was 7.86% (Table III). In fact, only GFP expressing lentiviruses were able to transduce the Jurkat cells and that was proportional to the viral amount used. The lentiviruses produced using the FUGW plasmid gave a transduction efficiency of 99.6%. Again, this confirmed that using 3 times more of Gag/pol is better for viral production and that is better to maintain the serum in the cell culture medium during viral production. The results also indicated that RFP might be toxic to the target cells and that the FUGW

lentiviral transfer vector might be a better option for viral production. Therefore, the FUGW plasmid was chosen for the subsequent experiments.

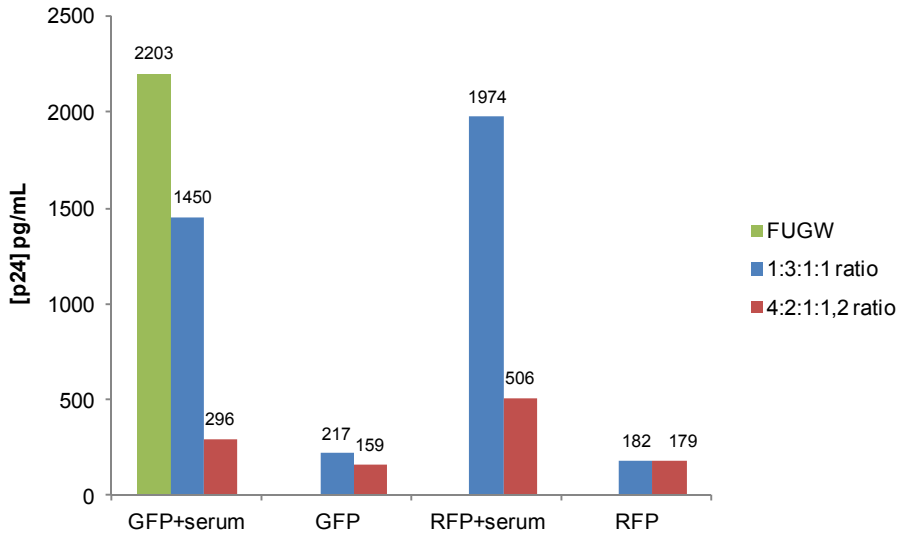


Figure 15. Titers of the VSV-G-pseudotyped lentiviruses encoding GFP or RFP, produced in the presence or absence of serum. 293T cells (5× 10^5 *per* well of a 6-well plate) were transfected by $CaPO_4$ precipitation with the plasmid ratios of either 1:3:1:1 (Transfer vector:Gag/pol:Rev:VSV) or 4:2:1:1,2 and in the presence or absence of serum in the medium. Each viral supernatant was harvested 48 hrs later and concentrated by ultracentrifugation before HIV p24 quantification. A viral sample obtained from other lab and produced with FUGW lentiviral transfer plasmid was used as a positive control and the concentration indicated corresponds to a dilution of 1:10 of that sample.

Table III. Transduction efficiency of Jurkat cells infected with the VSV-G-pseudotyped lentiviral vectors expressing GFP or RFP, produced in the presence or absence of serum and using different ratios of plasmids. Cells (4×10^4 *per* well of a 48-well plate) were infected with all the volume of lentiviruses through spinoculation for 1 hour at 2000 rpm and 32ºC. Three days later the percentage of reporter gene positive cells was analysed by flow cytometry.

RATIO	% FLUORESCENT CELLS				
	GFP + serum	**GFP**	**RFP + serum**	**RFP**	**FUGW**
1:3:1:1	7.86	2.57	0.00	0.00	99.6
4:2:1:1,2	4.42	2.09	0.16	0.11	

The influence of the transfection method in viral production was also assessed. The lipofectamine reagent and the $CaPO_4$ precipitation were used in parallel in the transfection using either the CMVΔ8.9 or the Gag/pol and Rev plasmids for comparison. In addition, other ratios of the plasmids were used in the experiment in which all the amounts of plasmids were increased, except the amount of the envelope

plasmid. According to Figure 16, higher viral titers were achieved by the $CaPO_4$ method when using the 3rd generation packaging constructs (Gag/pol and Rev in separate plasmids). Transduction of Jurkat cells was performed with spinoculation for 1:30 hours at 2200 rpm and 32ºC. Despite allowing a viral production two times lower than that achieved with lentiviruses generated by $CaPO_4$ transfection, the lentiviruses resulting from transfection with lipofectamine transduced Jurkat more efficiently (Table IV). This occurs probably because the $CaPO_4$ method produces more toxicity in the cells (cell detachment and death), releasing cell components that become co-concentrated with viral particles, thereby introducing toxic components to viral preparations. These results indicate that lentiviruses produced using lipofectamine allow superior transduction efficiency and further confirms that the 3rd generation lentiviral packaging vectors are better for producing high amounts of lentiviruses.

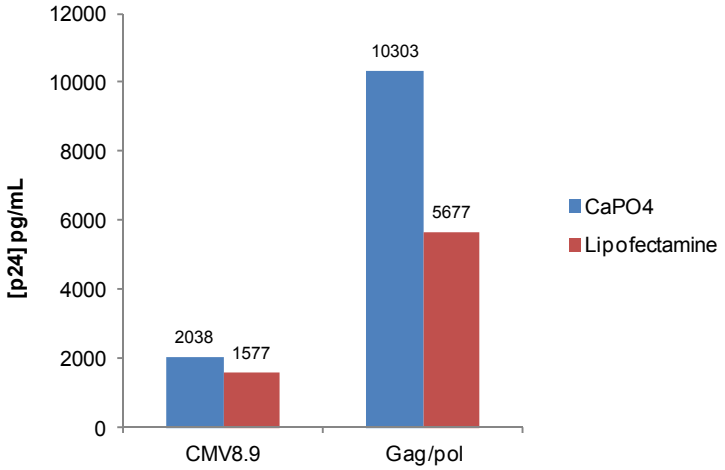


Figure 16. Titers of the VSV-G-pseudotyped lentiviruses expressing GFP (FUGW), produced either by $CaPO_4$ or by lipofectamine transfection. 293T cells (1×10^5 *per* well of a 24-well plate) were transfected either by $CaPO_4$ precipitation (with the plasmid ratios of 5:10:5:1 (Transfer vector:Gag/pol:Rev:VSV) or 10:10:1 (Transfer vector:CMVΔ8.9:VSV) or by lipofectamine (ratios of 3,2:10:3,2:1 or 10:10:1, for the four and three plasmid approach, respectively). Each viral supernatant was harvested 48 hrs later and subjected to HIV p24 quantification.

Table IV. Transduction efficiency of Jurkat cells infected with the VSV-G-pseudotyped lentiviral vectors expressing GFP produced either by $CaPO_4$ or lipofectamine transfection. Cells (8×10^4 *per* well of a 24-well plate) were infected with all the volume of produced lentiviruses through spinoculation for 1:30 hrs at 2200 rpm and 32ºC. Three days later the percentage of GFP positive cells was analysed by flow cytometry.

% GFP			
$CaPO_4$		Lipofectamine	
CMVΔ8.9	**Gag/pol**	**CMVΔ8.9**	**Gag/pol**
3.00	5.93	4.81	17.35

The use of a liposome-based reagent for transfection seems to greatly influence the transduction efficiency. Therefore, the Lipofectamine reagent was compared with Fugene 6, again using the tree-plasmid or the four-plasmid approach. Conditions for transfection and for transduction were maintained, except that this time new plasmid DNA was prepared and viruses were harvested 65 hours post-transfection. As it can be observed in Figure 17 and in Table V, virus collection at late time points after the initiation of vector production led to an increase in viral titer, and therefore, an increase in the transduction efficiency, compared with the previous result obtained by transfection with lipofectamine in which the viruses were harvested 48 hours post-transfection (Figure 16 and Table IV). For all the conditions tested, the transduction efficiency was proportional to the viral titer and was higher for the lentiviruses generated by Fugene 6 transfection, reaching 96.48% GFP positive cells.

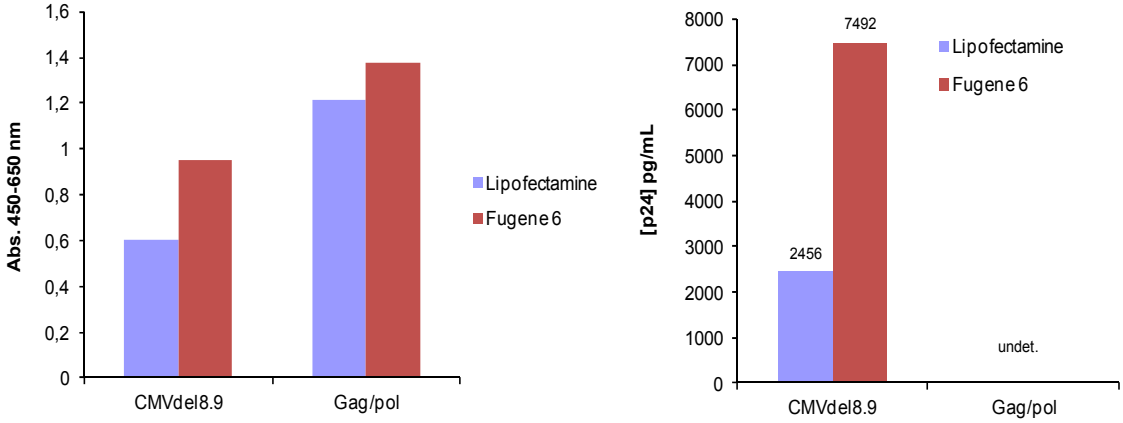


Figure 17. Titers of the VSV-G-pseudotyped lentiviruses expressing GFP (FUGW), produced either by Lipofectamine or by Fugene 6 transfection. 293T cells were transfected as mentioned on the previous assay and lentiviruses were harvested 65 hrs later for HIV p24 quantification. As the absorbance values for Gag/pol lentiviruses were too high (left graph) and outside the range of the calibration curve it was not possible to determine its concentration (right).

Table V. Transduction efficiency of Jurkat cells infected with the VSV-G-pseudotyped lentiviral vectors expressing GFP (FUGW) produced either by Lipofectamine or by Fugene 6 transfection. Cells (8×10^4 *per* well of a 24-well plate) were infected with all the volume of lentiviruses through spinoculation for 1:30 hrs at 2200 rpm and 32ºC. Three days later the percentage of GFP positive cells was analysed by flow cytometry.

% GFP			
Lipofectamine		**Fugene 6**	
CMVΔ8.9	**Gag/pol**	**CMVΔ8.9**	**Gag/pol**
23.36	84.35	60.88	96.48

Being determined the optimal conditions for viral production that allowed the highest transduction efficiency in Jurkat cells it was necessary to: 1) replace the GFP reporter gene in the FUGW lentiviral vector by a red fluorescent protein; 2) test again the transduction efficiency in Molt-4 cells and 3) use the Sindbis/anti-FITC envelope instead of the VSV-G. In Table VI are indicated the percentages of both Jurkat and Molt-4 transduction with VSV-G-pseudotyped lentiviruses expressing either GFP or RFP. The efficiency of Molt-4 transduction is clearly lower than that of Jurkat cells being more evident when the red fluorescent expressing lentiviruses were used. Taking into account this result, Jurkat cells were chosen to proceed with the further experiments for this project.

Table VI. Comparison of transduction efficiency between Jurkat and Molt-4 cells infected with the VSV-G-pseudotyped lentiviral vectors expressing GFP (FUGW) or RFP (FUW/RFP) produced using Fugene HD for transfection. Cells (8×10^4 *per* well of a 24-well plate) were infected with approximately 50 ng of lentiviruses through spinoculation for 1:30 hrs at 2200 rpm and 32ºC. Three days later the percentage of GFP or RFP positive cells was analysed by flow cytometry.

CELLS	% GFP	% RFP
Jurkat	99.38	76.89
Molt-4	24.15	1.51

Finally, the Sindbis/anti-FITC chimeric envelope was tested in another experiment, which included the VSV-G as positive control. Lentiviruses were prepared as previously mentioned and all the supernatant was used for infection after concentration by an AMICON filtration device. Table VII summarizes the results obtained for transduction. VSV-G/GFP lentiviruses gave a higher cell transduction (twice much) than VSV-G/RFP (92.2% versus 43.49%). Sindbis/anti-FITC/RFP, on the other hand, allowed only 7.66% Jurkat transduction. Therefore, it was still necessary to improve the efficiency of transduction.

Table VII. Transduction efficiency of Jurkat cells infected either with Sindbis/anti-FITC-pseudotyped lentiviruses expressing RFP or with VSV-pseudotyped lentiviral vectors expressing GFP or RFP. Cells (8×10^4 *per* well of a 24-well plate) were infected with approximately 500 ng of Sindbis/anti-FITC lentiviruses (concentrated by AMICON centrifugation) plus 1.5 μg/ml anti-CD7-FITC or with 40 ng of VSV-G lentiviruses through spinoculation for 1:30 hrs at 2200 rpm and 32ºC. Three days later the percentages of GFP or RFP positive cells were analysed by flow cytometry.

	% GFP	% RFP
Cells only	0.03	0.09
VSV-G/GFP	92.2	-
VSV-G/RFP	-	43.49
CD7-FITC	22.54	-
Sindbis/anti-FITC+CD7-FITC	42.11	7.66

There is a noticeable difference between the transduction efficiency mediated by lentiviruses expressing GFP and lentiviruses expressing RFP. Since the variation between the two lentiviruses resides only in the transgene, that may indicate that the RFP protein might cause some toxicity to the cells. A new variant of red fluorescent protein, called DsRed-Express2 but that will be here simplified as DsRed, had just

been engineered at the time and showed minimal cytotoxicity[207]. This new red fluorescent gene was cloned into the FUW transfer plasmid for the subsequent experiments. Transgene-related toxicity was also reported for a VSV-G-pseudotyped SIV expressing GFP[59]. When VSV-G-pseudotyped lentiviruses are used to transduce Jurkat cells at more than 200 ng of HIV p24 it become toxic to the cells and around 40/50% cell death can be observed, even using the less toxic DsRed gene.

Thus, another experiment was performed to produce lentiviral vectors pseudotyped with Sindbis/anti-FITC or VSV-G and expressing this time the new red fluorescent protein. Viruses were harvested 65 h later, concentrated by ultracentrifugation and used to transduce Jurkat cells. There was an improvement in Jurkat transduction with VSV/DsRed lentiviruses, which could now transduce 80% of the cells, even using an amount of viruses lower than the previously used for VSV/RFP (Table VIII). With the Sindbis/anti-FITC/DsRed lentiviruses the transduction efficiency was only 7.5%, despite the higher amount of virus used. As it will be shown in the next section, the *in vitro* transduction, some modifications were added which allowed us to overcome this difficulty in the transduction with Sindbis chimeric envelope.

Table VIII. Transduction efficiency of Jurkat cells infected with Sindbis/anti-FITC- or VSV-G-pseudotyped lentiviral vectors, both expressing DsRed. Cells (8×10^4 *per* well of a 24-well plate) were infected as described before with approximately 2.5 µg of Sindbis/anti-FITC lentiviruses (concentrated by ultracentrifugation) plus 5 µg/ml anti-CD7-FITC or with 25 ng of VSV-G lentiviruses. Three days later the percentage of GFP or RFP positive cells was analysed by flow cytometry.

	% FITC	% DsRed
Cells only	0.09	0.14
VSV-G/DsRed	-	80
CD7-FITC	68.2	0.15
Sindbis/anti-FITC+CD7-FITC	89.0	7.5

Therefore, from this set of experiments we can conclude that the ratio of the plasmids used to produce lentiviral vectors by transient transfection does influence viral titer. The four-plasmid approach using the 3rd generation packaging construct was more efficient for virus production than the 2nd generation. The amount of the Gag/pol plasmid should be higher than the other plasmids and the amount of the envelope

plasmid should be ten times lower than that one. This is in agreement with others that reported very small amounts of the envelope plasmid[9] and low ratio of envelope to transfer vector plasmid[38] for optimal infection and transduction efficiency. As will be seen early in the next point, a high amount of the lentiviral transfer plasmid is crucial. It is usually used more than twice the amount of the other plasmids[72]. The type of lentiviral transfer vector and its promoter-driven transgene expression can also influence the viral infectivity and the expression of the transgene. The FUGW construct has a better performance than the #318GFP-Fluc. The former contains the human ubiquitin-C promoter that proved to be stronger than the hPGK.

The presence of serum in the cell culture medium also favoured lentiviral production, and consequently infectivity, contrary to a study by Logan and colleagues[125] that observed superior vector production and a slightly elevated infectivity of vector produced in serum-free medium. Others have reported that lentiviral vector production in the absence of serum, while reducing immunogenicity, did not affected transduction efficiency[10].

The choice of the transfection reagent proved to be a determinant factor. A low toxicity liposome-based approach, like the Fugene or TransIT- LT1 (cheaper than Fugene but with the same transfection performance), is definitely the best to reach the highest viral titers and transduction efficiencies. The viral concentration method used is also important. We found that ultracentrifugation worked better than the AMICONs filter devices or the lenti-X concentrator (see Appendix C) as it allows a higher vector recovery.

Finally, the efficiency of targeting will obviously depend on the type of cells used. We found a significant difference between the levels of transduction of the Jurkat versus the Molt-4 leukemic cell lines using the same viral construct that is not due to differences in the levels of cell-surface labelling, as those assays were performed with VSV-G that does not need a targeting antibody as happens for Sindbis/anti-FITC.

3.1.6 In-vitro targeting of Jurkat cells

The effectiveness of the *in vitro* targeting of Jurkat cells can be assessed by producing Sindbis/anti-FITC pseudotyped lentivirus and transducing the cells with this virus. Transduced cells will express DsRed protein that can be detected by measuring the percentage of fluorescent cells through flow cytometry. Nevertheless, our transduction protocol needed further optimization. To do that it was used the Sindbis/ZZ envelope (that contains the Fc binding domain of protein A), whose transduction efficiency was already demonstrated[147], and two different groups of plasmid ratios for 293T cells transfection in order to produce virus. Moreover, transduction (with equal amounts of both groups of viruses) was carried out in plates coated or not with retronectin and with or without an acidic pH treatment that was performed approximately 18 hours later (Appendix B). It was previously demonstrated that the reduction of the pH in the culture medium following incubation of Sindbis virus with liposomes at 4ºC triggered fusion[198] and that cell lines resistant to transduction by a Sindbis ZZ mutant pseudotyped lentiviral vector showed an enhancement in infectivity of 20 to 25 fold with low pH treatment[150]. According to figure 46 (Appendix B), the lentivirus produced to higher titers, denominated "Black" (121 ng/ml *versus* 91 ng/ml of HIV p24 for the "Blue"), was not the one with the best efficiency of transduction, which benefited from retronectin and low pH treatment. These modifications to the protocol allowed us to obtain 85% transduction efficiency and thus, these were the conditions used to address the efficiency of the Sindbis/anti-FITC targeting system. Morizono and colleagues reported an efficiency of 77.85% in Jurkat transduction using a Sindbis ZZ envelope with mutations corresponding to our M3 and M4, and with similar conditions (low pH treatment, retronectin-coated plates, anti-HLA targeting antibody and 200 ng HIV p24 of lentivirus)[150].

A transduction assay was then performed using the anti-HLA-FITC antibody for cell labelling and using the Sindbis/anti-FITC Wt (without mutations in the envelope glycoproteins) and two Sindbis/anti-FITC envelope mutants M123 and M1234. These mutants showed less non-specific transduction when virus was added alone comparing with the Wt (Figure 18). In the presence of the HLA antibody, M123 showed the highest infectivity, although M1234 had less non-specific infection.

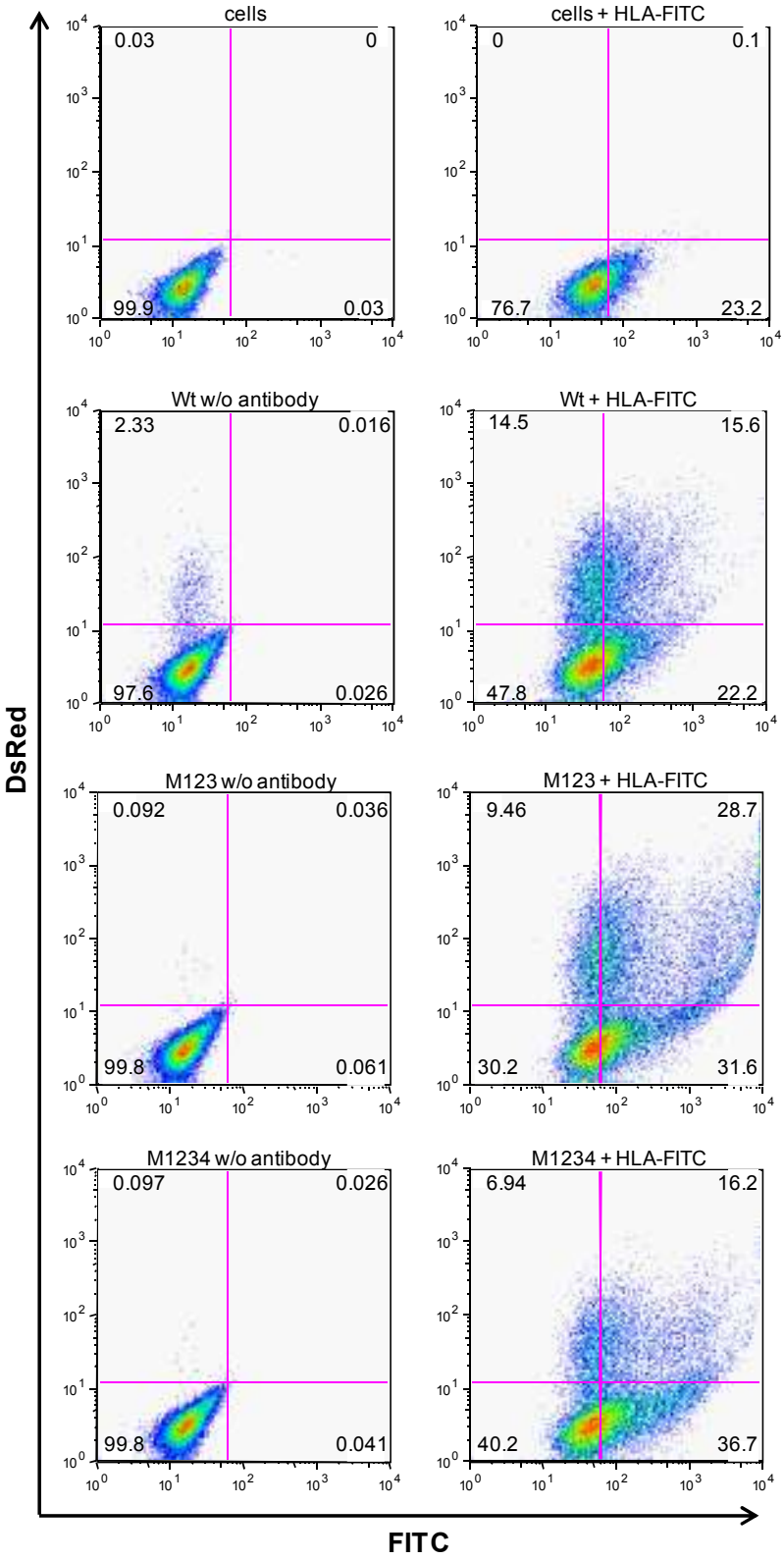


Figure 18. Transduction of Jurkat cells with the pseudotyped Sindbis/anti-FITC lentiviruses. Wt viruses and the mutants M123 and M1234 (~250 ng HIV p24) were spinoculated in a retronectin (60 µg/mL) coated 24-well plate and then cells and anti-HLA-FITC antibody (5 µg/mL) were added to the wells and spinoculated again. The next day cells were treated with a low pH buffer and medium was replaced. Fourty-eight hours later cells were analysed by flow cytometry for DsRed fluorescence.

In Figure 19A, it is shown a good efficiency of Jurkat transduction with Sindbis/anti-FITC Wt, which is higher when using the anti-CD7-FITC antibody for labelling the cells than when the anti-HLA-FITC is used (82.8% *versus* 55.8%, respectively). Moreover, with a lentivirus displaying a non-specific scFv (ST6 that binds to the CCR5 receptor[2]) at the surface of the wt Sindbis envelope there was no targeting to Jurkat cells, which indicates that the binding is mediated through specific binding of the FITC scFv to the FITC at the cell surface. However, using an antibody specific for B cells for cell labelling (anti-CD19-FITC) there was some non-specific targeting that was also observed when using the virus alone, meaning that the virus was able to enter the cells in a non-specific way. Compared with the results in Figure 18, one can say that the increase in transduction efficiency was accompanied by an increase in non-specific entry, and particularly when the virus is added alone rather than in the presence of the cell-coating antibody. In Figure 19B, it was investigated whether the mutants of the Sindbis envelope were able to reduce the non-specific transduction, this time using the anti-CD7-FITC antibody, and, in fact, that is observed with both the M123 and the M1234 Sindbis envelopes, but the effect is more visible with the lentivirus Sindbis/anti-FITC M1234 where the background is almost completely removed. Nevertheless, the infectivity is lower than the Wt lentiviruses (35.9% *versus* 64.8%). Results from both panels indicate that the inclusion of the receptor-specific FITC-conjugated antibody to which the scFv FITC can bind augments specific transduction while reducing non-specific one, thus reinforcing the fact that the presence of a targeting moiety can re-direct binding to specific cells. This further highlights the fact that insertion of the scFv did not block the folding of the entire envelope proteins or abrogated their fusion activity. Moreover, the results are in accordance with the ones obtained by Morizono and colleagues[152] for Sindbis/ZZ given that in the absence of antibody the background level of infectivity is reduced for the mutant viruses, particularly the Sindbis/anti-FITC M1234 (panel B), compared with the non-mutated envelope (Wt, panel A).

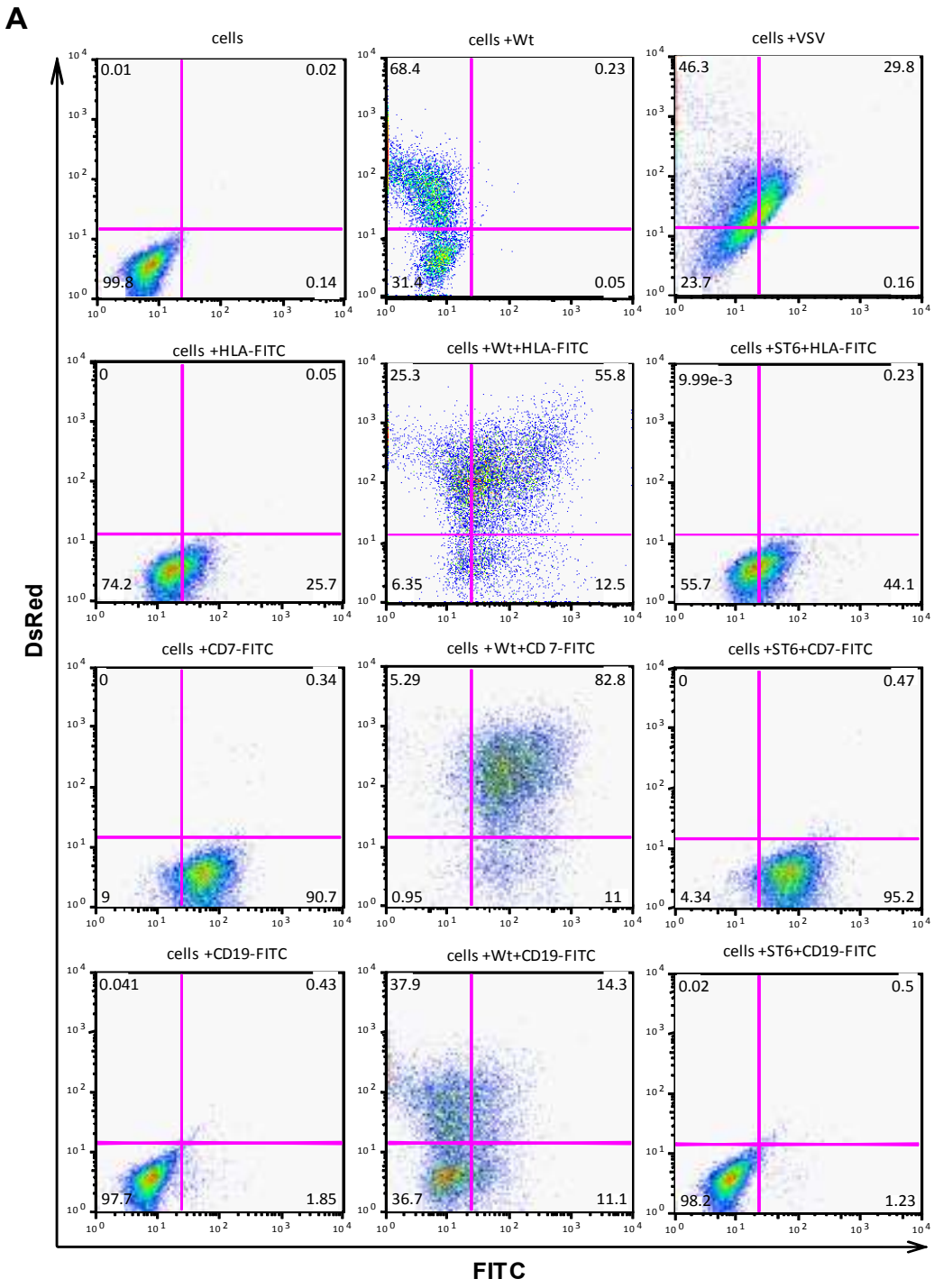


Figure 19. Transduction of Jurkat cells with the pseudotyped Sindbis/anti-FITC lentiviruses. Viruses were spinoculated in a retronectin coated 24-well plate and then cells and a FITC-conjugated antibody (5 µg/mL) were added to the wells and spinoculated again. The next day cells were treated with a low pH buffer and medium was replaced. Fourty-eight hours later cells were analysed by flow cytometry. In panel A are the results obtained with the non-mutated Sindbis envelope virus expressing the scFv anti-FITC (Wt) and another expressing the scFv anti-CCR5 (ST6), both using three different antibodies: anti-HLA-FITC, anti-CD7-FITC and anti-CD19-FITC. In panel B are shown the results for the Sindbis/anti-FITC Wt and mutants M123 and M1234 (same volume of virus) using the antibodies anti-CD7-FITC, anti-CD19-FITC and without antibody. In panel A, it was used 40 µg/mL of retronectin and in panel B, it was used 50 µg/mL.

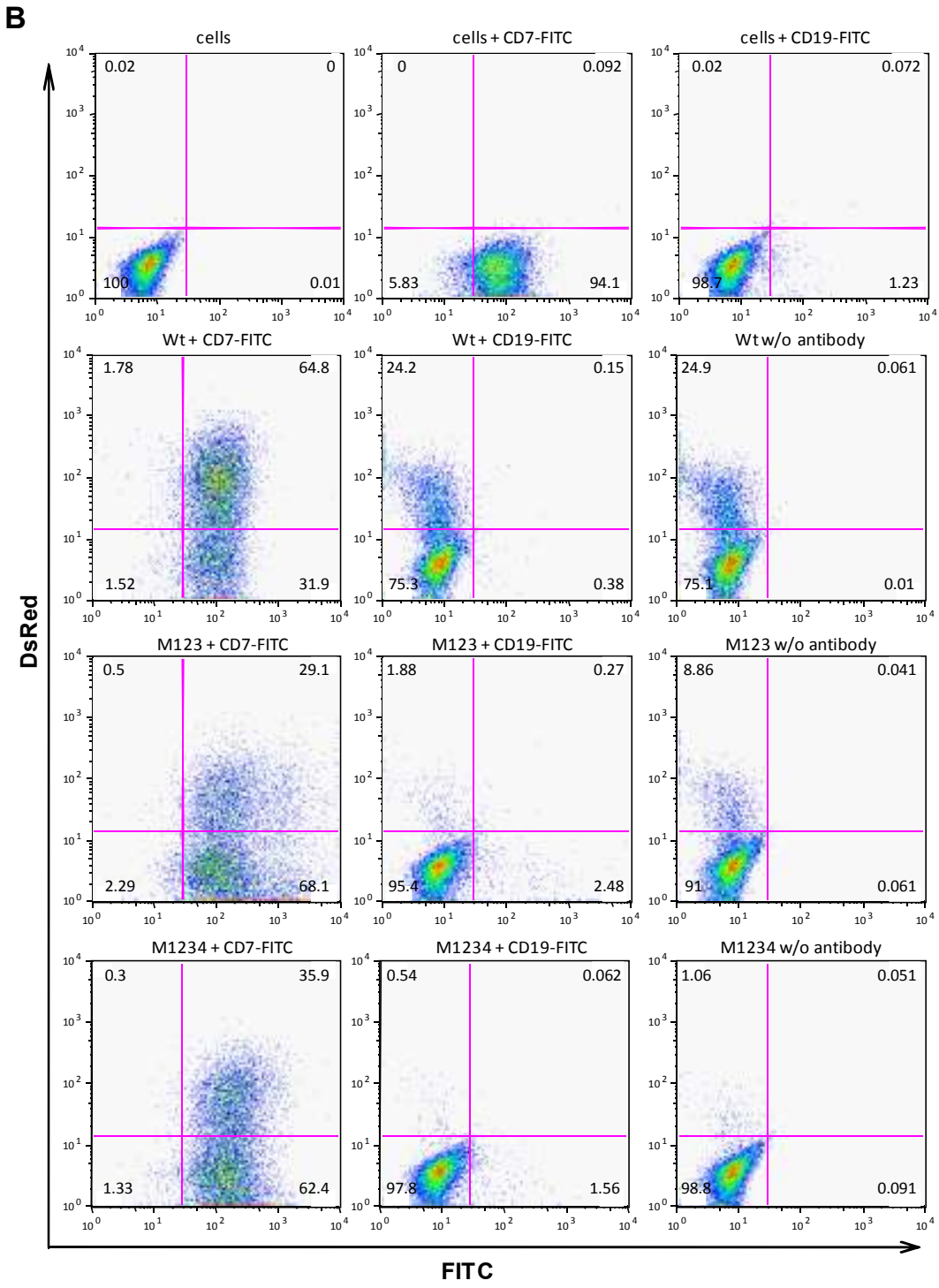


Figure 19. ***Continued.***

In summary, the difference in infectivity among the different Sindbis/anti-FITC mutants and Wt are due to differences in titer, as it was used the same volume of virus and not the same concentration, except for Figure 18. In figure 19B, where the viruses

were not normalized for HIV p24 concentration, one can see that the Wt allowed a higher transduction due to the production of higher titers than the other mutants did. Besides that, the variation observed for Wt within the three panels of experiments is also related with different amounts of retronectin used for coating the 24-well plates. The binding of the anti-FITC scFv to the FITC labelled cells mediated the viral entry, but when the ligand is not present, the virus (Sindbis/anti-FITC Wt) can enter via its natural receptors heparin sulphate and laminin, therefore it showed that levels of background transduction. Although viral vectors are extremely efficient in gene transfer, the alteration in target specificity of the virus can result in a decrease in infectivity of the recombinant virus and this explains the lower titer and infectivity of the Sindbis envelope mutants.

3.1.7 Competition of an anti-FITC antibody with the anti-FITC scFv displayed by Sindbis-pseudotyped lentiviruses

To demonstrate that the targeting is mediated by the anti-FITC scFv display we performed a competition assay where transduction was done in the presence of increased amounts of an anti-FITC antibody that would compete for binding to the FITC present on labelled cells. Targeted transduction decreased as the concentration of this soluble antibody increased, reducing transduction efficiency by 50%, whereas the isotype control antibody had no effect (Figure 20). This shows that the viral infectivity blockade is mediated by the antibody-specific inhibitory effects. Therefore, the specific targeting of FITC-labelled cells by chimeric scFv-Sindbis pseudotyped lentiviral vectors is triggered by the recognition and binding of the anti-FITC scFv to the FITC.

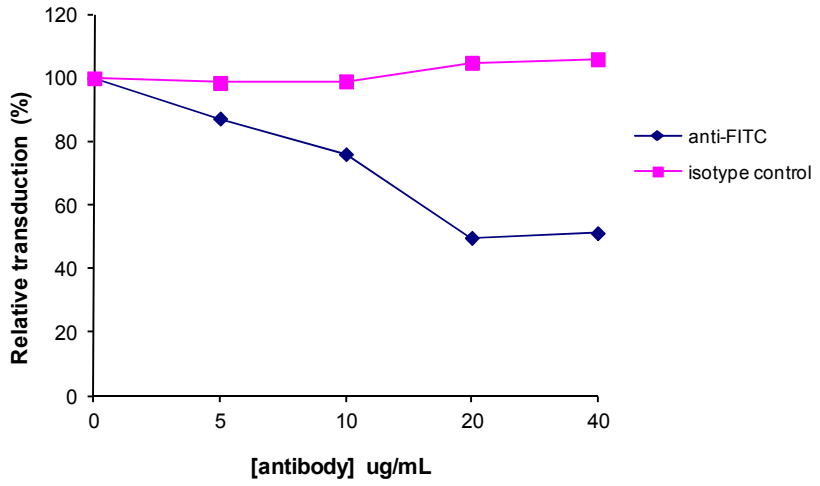


Figure 20. Competition between an anti-FITC antibody and the anti-FITC scFv displayed by Sindbis-pseudotyped lentiviruses for binding to FITC-labelled Jurkat cells. Effect of the addition of a soluble anti-FITC antibody on the efficiency of targeted transduction. Increasing concentrations of anti-FITC or isotype control antibody were added to the wells during transduction, which was performed as described before. On the following day, medium was replaced with a new one after a low pH treatment and DsRed expression (transduced cells) analysed 2 days post-transduction by flow cytometry. Values are presented as the percentage of transduced cells relative to the control without soluble anti-FITC antibody. This figure is a representative of two independent assays.

3.1.8 Suicide gene therapy

From the wide range of suicide genes employed in gene therapy, DT-A and HSV/TK are among the most successfully used and for this reason both strategies were chosen to be tested.

3.1.8.1 The DT-A approach

A single molecule of diphtheria toxin is sufficient to kill a cell[238], what makes it a highly potent strategy. Therefore, the initial approach adopted to kill the leukemic cells was the DT-A. DT has two subunits: the A subunit that contains the catalytic activity and the B subunit that binds to receptors present at the cells surface[46]. DT-A inhibits protein synthesis by catalysing the ADP-ribosylation of diphtamine, a post-translationally modified histidine residue, in elongation factor-2 (EF-2)[46], and triggers apoptosis and detachment of cells[35]. The DT-A gene was cloned in the lentiviral vector FUW and DT-A toxicity from this construct was tested by measuring its ability to inhibit protein synthesis (Figure 21), which was compared to other plasmids available in the lab that were used as controls, PSA-DTA, containing a prostate-specific antigen (PSA) promoter, and ROSA-DTA, allowing expression in a ubiquitous manner. Two different cells lines, 293T and HeLa, were used because of the possible differences that might be observed from cell to cell using different promoters. Cells were co-transfected with the DT-A plasmids and a control plasmid with the firefly luciferase gene under the UbiC promoter. Cells were harvested 65 h post-transfection and luciferase activity was measured in the protein extracts. Reduction in luciferase activity was used as a measure of the inhibition of protein synthesis. The FUW-DTA construct revealed to be as efficient as the other ubiquitous expressed construct in inhibiting luciferase expression (Figure 21). On the other hand, DT-A expression from the tissue-specific promoter plasmid was able to reduce the luciferase expression in the HeLa cancer cell line but did not cause changes to luciferase expression in 293T cells. These results demonstrated that this construct was completely functional.

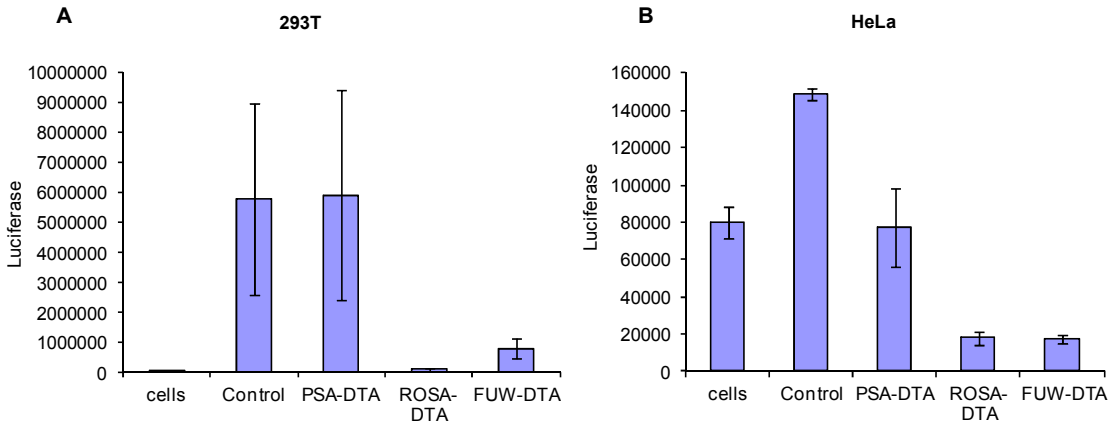


Figure 21. Inhibition of protein synthesis by FUW-DTA in 293T (A) and HeLa (B) cells. Cells were transfected with a plasmid expressing firefly luciferase (FUW-Luc) either alone (control, 0.8 µg) or co-transfected with other plasmids expressing DTA (0.1 µg). The total amount of plasmid was maintained constant by addiction of an empty plasmid. Sixty-five hours post-transfection cells were harvested and subjected to a luciferase assay. PSA-DTA and ROSA-DTA were used as positive controls for DTA inhibition. Y-axis indicates absolute luciferase values. Error bars show standard deviation.

Because the toxin is extremely toxic, its expression in the virus-producer cells will inhibit protein synthesis and, consequently, viral production (for the generation of viruses containing DT-A) will be negatively affected. For that reason, a DT-A-resistant cell line was employed to try to overcome this difficulty in generating lentiviral vectors (non-tissue specific) containing the DT-A toxin gene in 293T cells. 293DTR cells[119] are resistant to apoptosis mediated by the toxin because it has a mutation in EF-2 that blocks ADP-ribosylation by the DT-A toxin [104] (in these cells, the toxin is incapable of EF-2 inactivation and can support the growth of viruses expressing the toxin). To confirm the reduced DT-A inhibition of protein synthesis in the new cells it was performed again a luciferase assay (Figure 22). Only a slightly decrease was observed in the luciferase expression levels.

The efficacy of the DT-A toxin was further tested by observing a reduction in DsRed fluorescence (Figure 23). In comparison to 293T cells, in which the expression of DT-A has a dramatic reduction in the fluorescence levels, in 293DTR cells the toxin basically has no effect. Moreover, these results also confirm that these DTA-resistant cells do not allow a high level of gene expression.

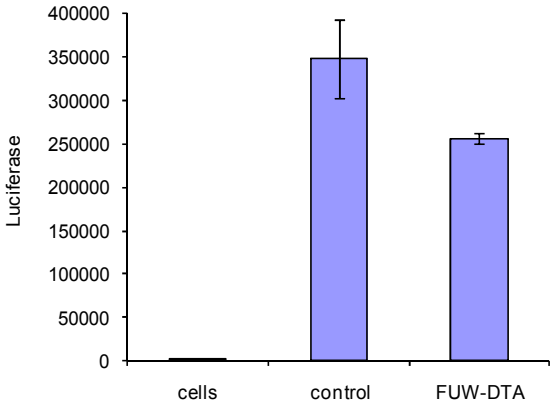


Figure 22. Inhibition of protein synthesis by FUW-DTA in 293DTR cells. Cells were transfected with a plasmid expressing firefly luciferase (FUW-Luc) either alone (control, 0.8 µg) or co-transfected with FUW-DTA (0.1 µg). The total amount of plasmid was maintained constant by addiction of an empty plasmid. Sixty-five hours post-transfection, cells were harvested and subjected to luciferase assay. Y-axis indicates absolute luciferase values. Error bars show standard deviation.

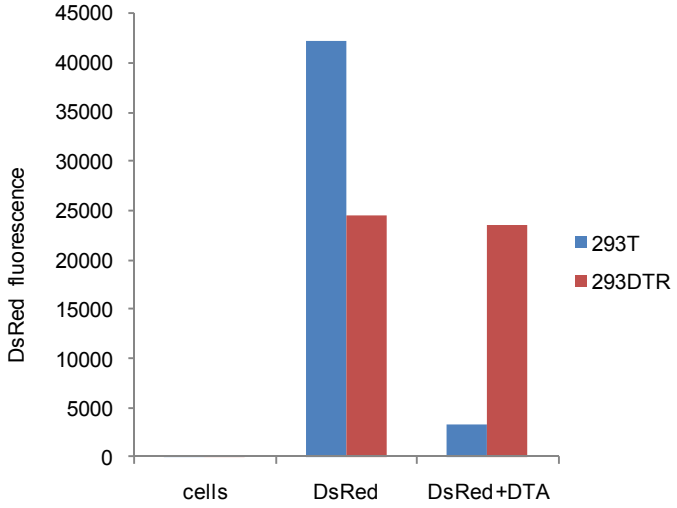


Figure 23. Inhibition of protein synthesis by FUW-DTA in 293T and 293DTR cells. Cells were transfected with a plasmid expressing DsRed (FUW-DsRed) either alone (DsRed, 0.8 µg) or co-transfected with FUW-DTA (0.1 µg). The total amount of plasmid was maintained constant by addiction of an empty plasmid. Sixty-five hours post-transfection, cells were harvested and red fluorescence was quantified. A reduction in DsRed expression was observed in the presence of DT-A but only in the sensitive 293T cells. In 293DTR it had almost no effect. This is a representative of two independent assays.

Thus, the 293DTR cells were transfected with the vectors needed for viral production, but still, the viral titer of the supernatant harvested from the cells was very low (3.2 ng/ml) compared with the one usually obtained for virus produced in 293T cells (>50

ng/ml), indicating that possibly no viral particles were being generated. The amount of plasmid used for transfection (1.4 µg) was still toxic to the 293DTR cells. This could indicate that the cells were only resistant to low amounts of the toxin (partially resistant to the toxin). However, in the study first describing this cells, they tested the sensitivity to DT holoenzyme at concentrations ranging from 10^{-11} M to 10^{-7} M (this maximum corresponds to ~ 6-7 µg/mL) and 293DTR cells were resistant to 10^{-7} M. One possible explanation for the DT-A sensitivity of the cells in our hands would be that the EF-2 mutation had reverted to wild type.

Therefore, it was adopted another strategy to overcome the problem with virus production, which consisted in the addition of nicotinamide during viral production that at high doses reverses the ADP-ribosylation of EF-2[146]. For that, 293T cells were co-transfected with the plasmids needed for lentiviral production of VSV/DT-A and 4 hours later different amounts of nicotinamide were added (0, 1, 4 and 8 mM). The supernatant was harvested and titrated but, again without success in virus production. In parallel, cells were transfected to produce VSV/DsRed generating a good viral titer.

Thus, all these strategies to produce lentiviral vectors expressing the DT-A were unsuccessful. Possibly, a better approach to overcome the toxicity of DT-A would be to control its expression during the phase of viral production. That can be done at the transcriptional level using promoters (for example, tissue-specific promoters) that would be turned off in the virus-producer cells but that could be activated in target cells. That has been achieved for instance using the PSA promoter to target DT-A gene expression to prostate cancer cells[242, 244, 119] although, in some cases, the toxicity was not completely eliminated even when using attenuated versions of the toxin[97] . In addition, a synthetic beta-catenin-dependent promoter (CTP4) that allowed the generation of adenovirus vectors expressing DT-A showed promise for gene therapies of tumours deregulated for beta-catenin[121]. This strategy could have been applied to our lentiviral delivery system if T-cell specific promoters were available. Another approach would be to use a regulatable or inducible promoter in the vector system.

Nanoparticles can be an alternative to viral vectors to deliver DT-A and avoid the problems associated with its toxicity in virus-producing cells. For instance, the lab of Dr Janet Sawicki, who gently provided the original DT-A plasmid, has been using polymeric nanoparticles to deliver DT-A combined with transcriptional regulation to target gene expression to prostate and ovarian tumours[166, 86]. Nevertheless, more recently, Wang and colleagues created a DT-A-resistant 293T cell line that allowed a non-integrating Rev-dependent lentiviral vector carrying DT-A and human TRAF6 to target HIV-positive cells[231].

The advantage of DT-A gene for suicide gene therapy is that it does not need the administration of a prodrug. Additionally, an efficacious concentration of the prodrug is not always achievable for *in vivo* applications and yet some cells that contain TK might be resistant to GCV killing[80]. Nevertheless, given the problems in the production of virus, we had to abandon this option for our suicide gene therapy and focus on the HSV/TK strategy.

3.1.8.2 The HSV-TK approach

To determine if the HSV-TK/GCV suicide gene strategy would work with our Sindbis/anti-FITC lentiviral vector to kill specifically the target cells, a spliced form of the TK gene (TK.007)[175] was cloned into the lentiviral vector FUW. Because of the possible problems associated with the positioning of the TK gene in the lentiviral vector plasmid[14], three different constructs were designed to further evaluate expression and choose the best one for our purpose. The goal was to obtain a good expression of both transgenes, DsRed and TK, cloned into the FUW backbone. In two of the constructs, the transgenes are in fusion: DsRedTK and TKDsRed. In the third vector, TK expression is internal ribosomal entry site (IRES)-dependent: DsRedIRESTK. These plasmids were transfected in 293T cells and analysed for DsRed, 65 hrs post-transfection, and for AlamarBlue fluorescence intensity, 5 days post-transfection and in the presence of 1 µg/mL of GCV (Figure 24). Although TK expression was quite similar among them, FUW/DsRedIRESTK showed the highest expression of DsRed, and was the construct chosen for this suicide gene approach. Of notice is the fact that GCV, at that concentration, was not toxic to untransfected 293T cells.

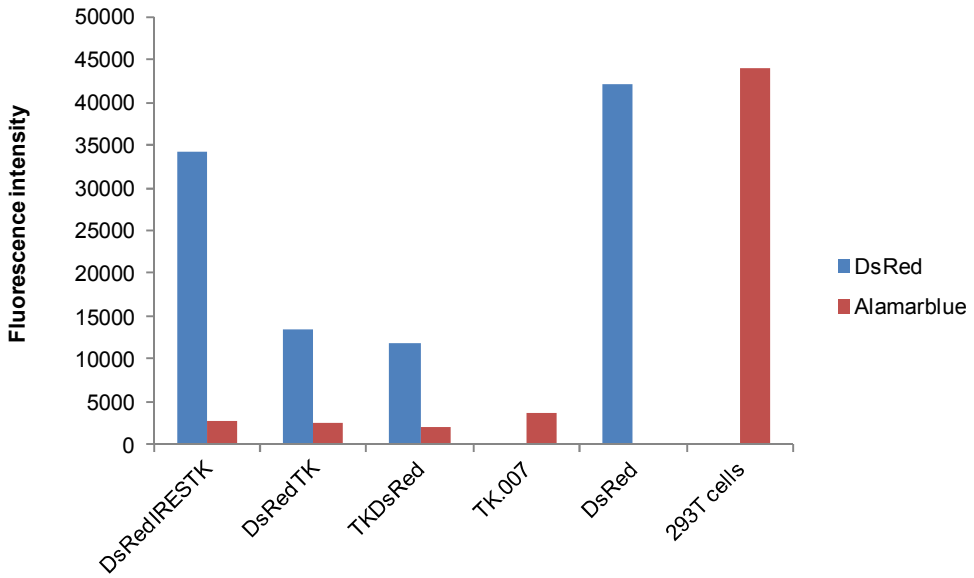


Figure 24. Fluorescence intensity of the DsRed and TK constructs. Cells were transfected with the indicated FUW plasmids and subjected to GCV treatment (1µg/mL) for 5 days, after which cell viability was analysed by alamarblue fluorescence. DsRed fluorescence was measured approximately 65 hours post-transfection. TK.007 and DsRed plasmids were included as controls.

To perform a titration of the GCV concentration, 293T cells were transfected with the FUW/DsRedIRESTK plasmid and, on the following day, it was added different concentrations of GCV, above and below the one initially used. Cell viability was measured by alamarblue assay, 5 days after transfection. A decrease in cell viability was observed with a GCV concentration as low as 0.25 µg/mL (Figure 25).

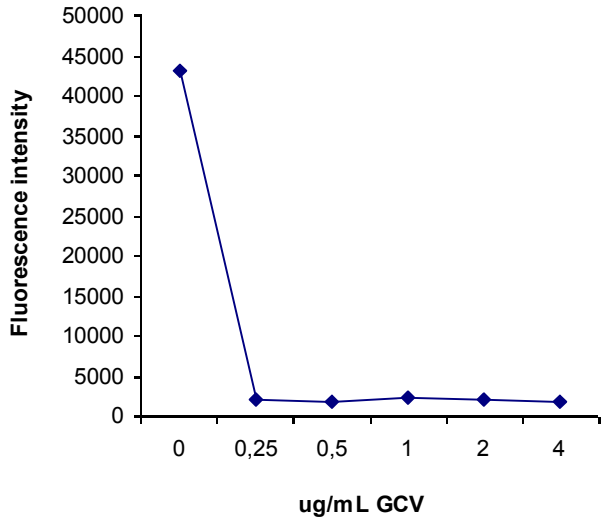


Figure 25. GCV sensitivity of 293T cells expressing DsRedIRESTK assessed by Alamarblue assay. Cells were transfected with the FuW/DsRedIRESTK plasmid and treated with different concentrations of GCV for 5 days, after which cell viability was analysed by Alamarblue assay. This is a representative of two independent assays.

Furthermore, to confirm TK gene expression and DsRed expression, another experiment was carried out where FUW/DsRedIRESTK transfected cells were treated with the same increasing concentrations of GCV over a period of 5 days, after which they were analysed for DsRed expression by flow cytometry (Figure 26). DsRed positive cells were plotted for forward and side scatter thus allowing to distinguish live and dead cell populations. The killing effect of HSV-TK/GCV was demonstrated by a clear reduction in the percentage of live $DsRed^{+}$ cells (from 79.9% to 4.86%).

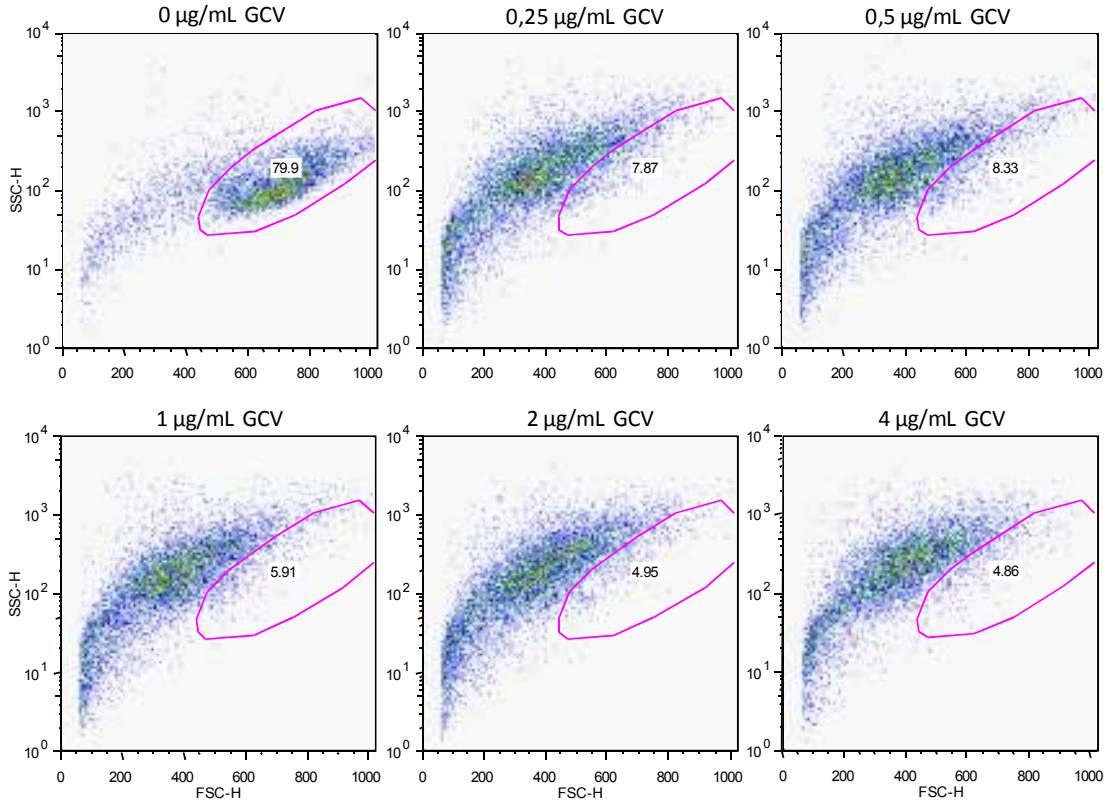


Figure 26. GCV titration in 293T cells expressing DsRedIRESTK by flow cytometry. Cells were transfected with the FUW/DsRedIRESTK plasmid and treated with different concentrations of GCV for 5 days, after which cells were analysed by flow cytometry. Cells were gated for DsRed and the DsRed$^+$ cells were then plotted for forward and side scatter. Specific killing was assessed by observing a reduction in the percentage of live DsRed$^+$ cells.

While this system is functioning well in 293T cells, it would be important to verify that the same is true in Jurkat cells. VSV-G-pseudotyped viral vectors expressing this construct were used to transduce Jurkat and titrate the amount of GCV needed to kill the cells, again over a period of 5 days (Figure 27). GCV treatment started on the day after transduction and whenever the cells needed to be split, a new medium with the respective concentration of GCV was added. One can observe that an amount as low as 0.25 µg/mL was already enough to kill most of the cells (88%). According to these results, the concentration of GCV chosen to be used in further experiments was 1 µg/mL, that allowed 91.6% of cell killing.

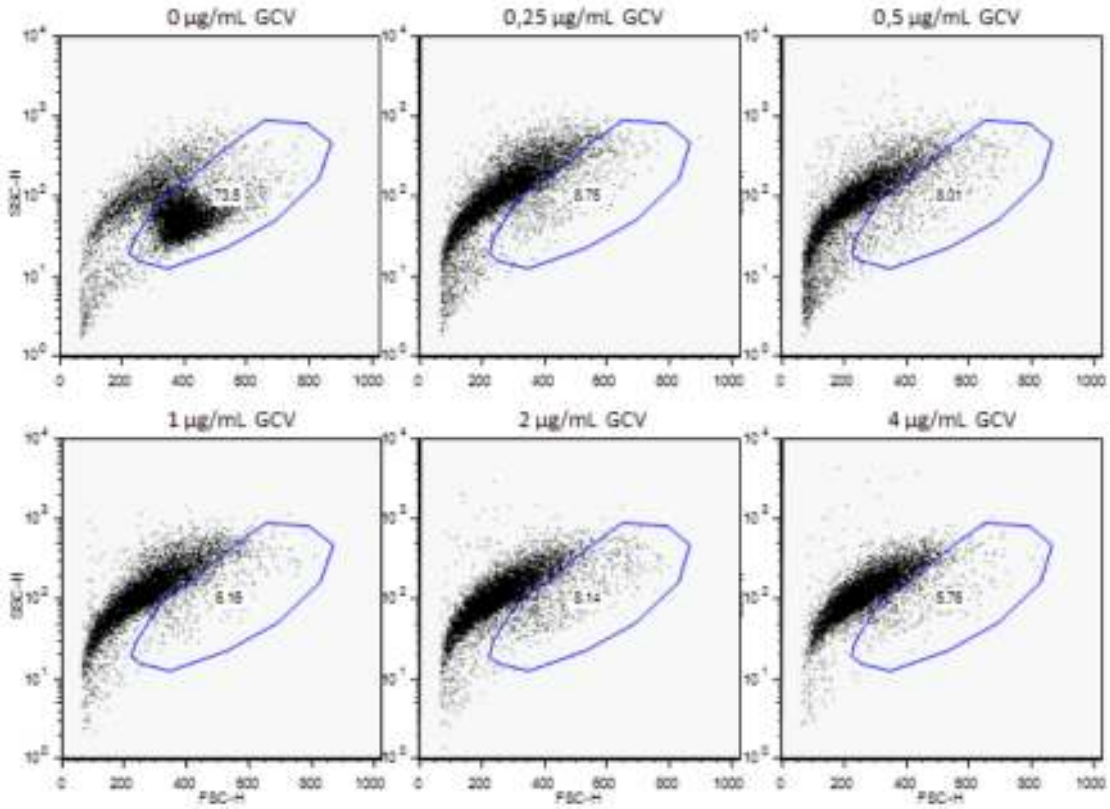


Figure 27. GCV titration in Jurkat cells expressing DsRedIRESTK. Jurkat cells were transduced with VSV-G-pseudotyped lentiviruses expressing DsRedIRESTK and treated with the indicated concentrations of GCV during a period of 5 days, after which they were analysed by flow cytometry. Cells were gated for DsRed^{+} (transduced) and this population was then plotted for forward and side scatter. The killing was assessed by observing a reduction in the percentage of live DsRed^{+} cells.

Finally, it was performed a targeting experiment using pseudotyped Sindbis/anti-FITC M1234 lentiviruses expressing DsRedIRESTK. Viruses were produced and Jurkat cells transduced, using the same conditions as for the assays on the previous section. After the low pH treatment, medium was replaced by another containing 1µg/mL of GCV, over a period of 12 days. Specific killing was assessed by observing a reduction in the percentage of DsRed positive cells (transduced cells), by flow cytometry analysis. Several time points were performed for analysis of the transduced cells over the time period of GCV treatment and a gradual reduction in the percentage of DsRed positive cells was observed up to day 8, after which it was maintained at 5% transduced cells (Figure 28). Therefore, these results demonstrate that Sindbis/anti-FITC M1234 lentiviruses can kill specifically and efficiently the target cells, although not completely, since a small number of cells remain unaffected. An explanation for this can be that the GCV dose may be insufficient for total eradication or, that it only acts

upon dividing cells and some of the transduced cells might be growth suppressed. The existence of cryptic splice sites is not an issue in our case, as HSV-TK (TK.007) is a new gene variant devoid of splicing sites. There was no unspecific killing of the non-transduced population due to GCV treatment, for each time point analysed (see Appendix F for analysis corresponding to day 8).

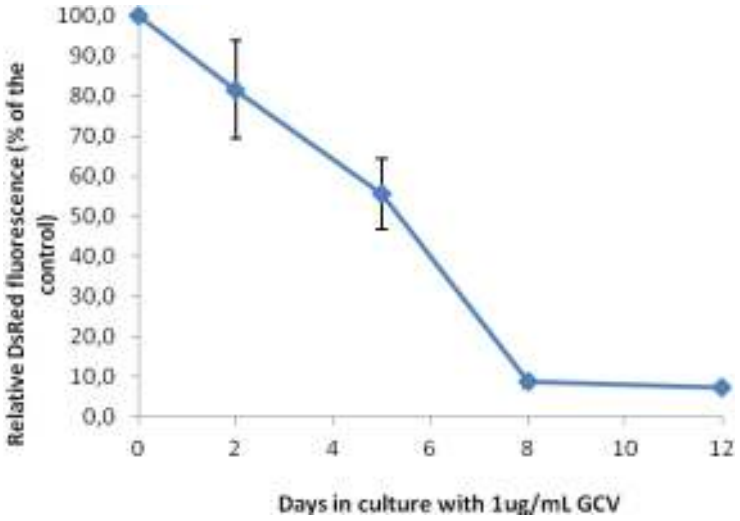


Figure 28. Ganciclovir treatment can kill transduced Jurkat cells expressing DsRed. Jurkat cells were transduced with pseudotyped Sindbis/anti-FITC M1234 lentiviruses expressing DsRedIRESTK and treated with 1µg/mL GCV during 12 days. Specific killing was assessed by observing a reduction in the percentage of $DsRed^+$ cells (transduced cells). The percentage of DsRed fluorescence is shown relative to the control (transduced cells without GCV treatment). Assay was performed in triplicate and error bars indicate the standard deviation.

Alternative splicing site in the TK gene, resulting in the expression of a non-functional TK in the minority of transduced cells, was a major limitation in clinical trials with TK cells. The HSV-TK employed in this work was already tested in clinical trials[42]. The activity of this new suicide gene was superior to the conventional HSV-TK, mediating considerably faster and higher absolute killing at low GCV concentrations, with reduced nonspecific toxicity, when used for donor-lymphocyte modification in adoptive immunotherapy models[175]. A recent report has indeed demonstrated an improved anti-tumour activity of TK.007 and a considerably stronger bystander effect as compared to conventional HSV-TK [174].

The immunogenicity of viral-derived TK protein can be also a problem, however when TK cells are infused to immunosuppressed patients they are well tolerated and persist

in the long term[217, 139]. In our model system, this immunogenicity would not be a problem given that it will be used immundeficient animals.

Although we used an IRES linking the two transgenes, most of the studies that used a HSV-TK suicide gene therapy used a fusion between the TK and the other reporter gene (for example,[126]). The fusion between reporter gene and TK would have an advantage, enabling to confirm that the elimination of cells resulted directly from TK action and not spontaneous death due to prolonged culture. Most of the vectors developed have the TK gene cloned as C-terminal fusion to EGFP gene[161, 85]. Nevertheless, Bennour and colleagues[14] reported expression of only one of the genes (CD34) in a fusion between tCD34 and cHSV-TK because of a posttranslational effect leading to breakage of the fusion protein, which renders the cells resistant to GCV. In our case, the construct DsRedIRESTK allowed higher cap-dependent expression of DsRed than the fusion DsRedTK, but similar levels of TK expression. Fehse and coworkers reported an unexpected higher expression of TK from the second position in an IRES vector as compared with the fusion protein (in Jurkat cells)[66]. On the contrary, in another study, lower expression was found when the gene was under IRES-dependent expression than when under cap-dependent expression (LTR-driven)[145].

Hoggarth and colleagues [85] obtained a reduction to 4% in viable GFP^+ Herpesvirus saimiri transduced Jurkat cells when exposed to 100 ng/ml of GCV for 7 days, whereas in our Sindbis/anti-FITC lentiviruses system, $DsRed^+$ cells were reduced to 5% in 12 days using 1000 ng/ml GCV. Nevertheless, we had observed that 250 ng/ml, the minimum GCV concentration tested, was enough to get essentially the same proportion of cell death and besides that they used a different viral vector with a high multiplicity of infection (MOI of 100), while we used an MOI of approximately 20

3.2 In vivo gene therapy

The *in vitro* results shown in the previous section indicated that this strategy of gene therapy could target specifically leukemic target cells in culture and deliver the transgene. These results provided us enough, and promising, evidence to move forward with this gene therapy model, i.e., to move to pre-clinical tests. To demonstrate that our strategy has potential for *in vivo* applications it was used in a mouse model of leukaemia. The chimeric Sindbis/anti-FITC envelope employed in this part of the work was the mutant M1234, which has a reduced non-specific transduction *in vivo*, as also demonstrated by others[152].

3.2.1 The choice of gene reporter (transgene) for detection of transduced cells

To evaluate transduction *in vitro* (gene transfer efficiency), we have detected the percentage of $DsRed^+$ cells. To check if the same marker could be used for the *in vivo* studies, its expression had to be tested in a CCD camera (IVIS Lumina) that is widely used for *in vivo* imaging, particularly for the detection of bioluminescent signals. Supposedly, the red fluorescence can also be assessed in this camera. Therefore, Jurkat cells expressing DsRed were prepared by transduction with VSV-G-pseudotyped lentiviruses (VSV-G/DsRed) with an efficiency of 99%[1]. Serial dilutions of Jurkat/DsRed were prepared in a 24-well plate and red fluorescence was detected under the CCD camera (Figure 29), with a strong signal corresponding to 1×10^7 cells. Given that it was possible to detect that signal in a culture plate, the next step was to check if it could also be detected in a mouse. Ten million Jurkat/DsRed cells were inoculated subcutaneously on the right and then on the left side of a Balb/c mouse but no red fluorescence was detected, except for a non-specific "spot" on the right side (Figure 30). Actually, most of the *in vivo* studies using a CCD camera for real-time whole body imaging rely on bioluminescent proteins, as a biofluorescent marker requires a higher level of expression for IVIS Lumina detection, as it is less sensitive, compared with bioluminescent luciferases. Curiously, the studies using this type of instrumentation for imaging with fluorescent proteins are performed in nude mice (for example [228]), as fluorescence imaging of cancer cells do not produce good images due

[1] Cells were 96% $DsRed^+$ after 1 month in culture.

to significant autofluorescence caused by the presence of hair [179]. Moreover, cells are usually injected subcutaneously due to low tissue penetration of the fluorescent light. Therefore, it is not surprising that we have not observed any signal in the mouse.

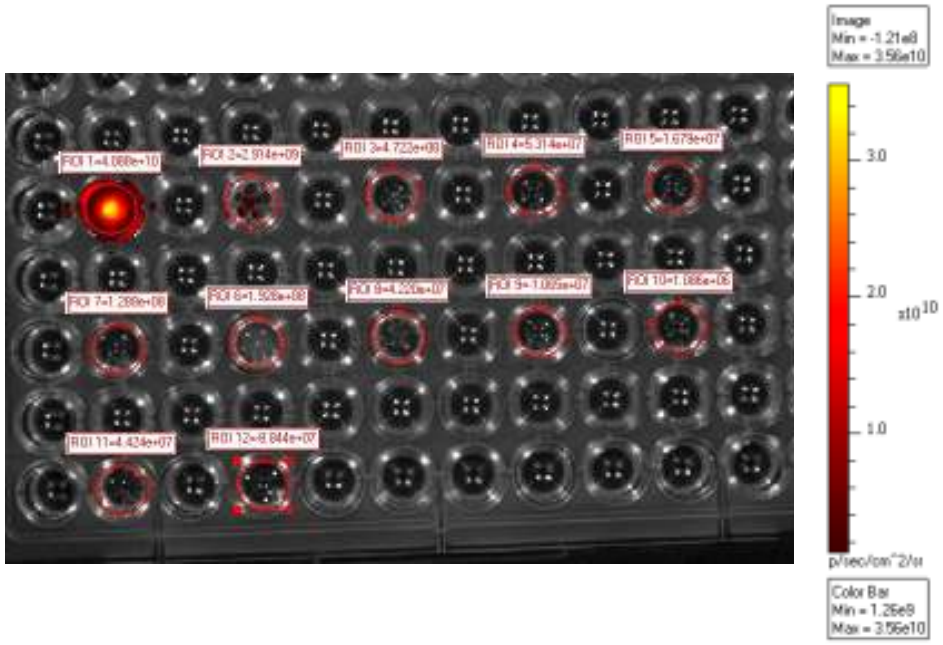


Figure 29. Imaging of fluorescence of Jurkat/DsRed cells in a cell culture plate. Several tenfold dilutions of Jurkat/DsRed cells, ranging from 1×10^7 to 100, were plated on a 24-well plate, followed by Jurkat cells, at the same range of dilutions, used as a control. Fluorescence was detected in a CCD camera. The p/sec/cm^2/sr represents photons/sec/cm^2/steridian.

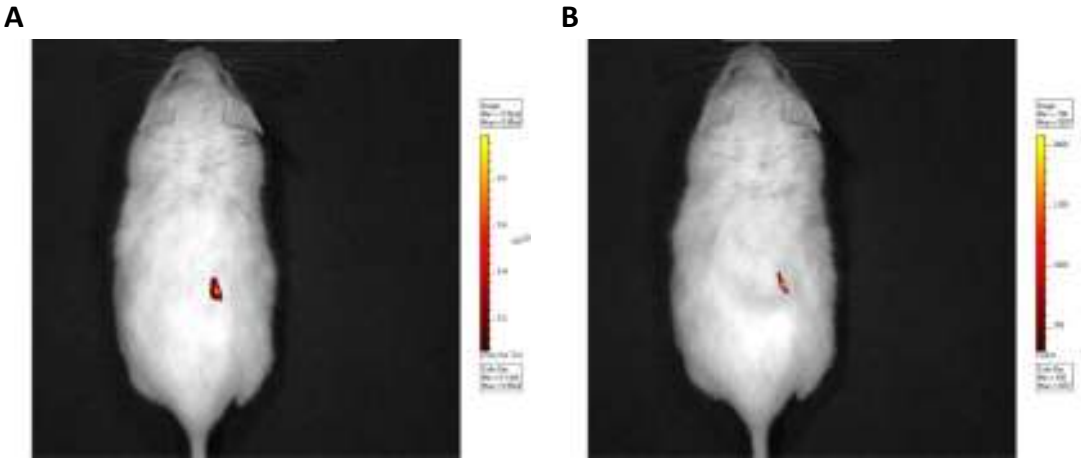


Figure 30. Imaging of fluorescence of Jurkat/DsRed cells in a Balb/c mouse. Ten million cells were injected subcutaneously on the right side (A) and on the left side (B) of a Balb/c mouse and imaged on the IVIS Lumina.

Thus, the option was to choose also bioluminescence to detect the transduction efficiency, in addition to the engraftment of leukemic cells. This is possible using two luciferase proteins, firefly and renilla, the former for cell detection and the latter for transduction, which have different substrates for luminescence detection. Therefore, it was generated the construct FUWDsRedIRESRenilla, that would allow detection of transduced cells either by real-time live imaging on a CCD camera (to detect renilla luminescence) or by flow cytometry of cells removed from organs (to detect DsRed fluorescence).

3.2.2 Establishment and validation of the animal model for cell engraftment

The immunodeficient NOD/SCID mouse was shown to be highly receptive to engraftment of primary childhood ALL cells[203, 20] and appear to retain the phenotypic and genotypic characteristics of the original patient sample[20]. The NOD/SCID xenograft mouse model is one of the most successful models to study ALL in which patient bone marrow leukaemia cells are directly transplanted into NOD/SCID mice[123]. The kinetics of engraftment reflects the human disease, leading to bone marrow infiltration, followed by migration to the spleen, peripheral blood and other haematopoietic organs[158, 123]. They have impaired T- and B-cell lymphocyte development, impaired natural killer (NK) cells and no complement activity, which facilitates xenografts[197]. Therefore, the animal model initially planned for the *in vivo* work was the NOD/SCID mice engrafted with MOLT-4 leukemic cells expressing GFP-Fluc to facilitate the detection of the engraftment. However, we have observed that Molt-4 cells are not as efficiently transduced as Jurkat cells either with VSV-G lentiviruses (Table VI from section 3.1.5) or with Sindbis/anti-FITC/DsRed lentiviruses (15% for Molt-4 *versus* 52% for Jurkat, Appendix D). Therefore, and given that the *in vitro* targeting assays were performed in Jurkat cells, we would have to use an animal model for T-ALL based on Jurkat cell engraftment. In parallel with the injection of 6 NOD/SCID mice with Jurkat/GFP-Fluc, 4 mice were also injected with Molt-4/GFP-Fluc, both with 20 million cells and through the tail vein. Four NOD/SCID mice died immediately after Jurkat/GFP-Fluc injection. One week later, the mice that survived were visualized on the IVIS Lumina to verify cell engraftment (one NOD/SCID/Molt-4/GFP-Fluc mouse died during anaesthesia) but only the Molt-4/GFP-Fluc injected NOD/SCID mice engrafted, as showed by the luminescent signal in the femurs (Figure 31). After 5 weeks there was no signal detected in the two Jurkat/GFP-Fluc injected NOD/SCID mice that had survived, both by the IVIS Lumina and by flow cytometry of peripheral blood cells (data not shown).

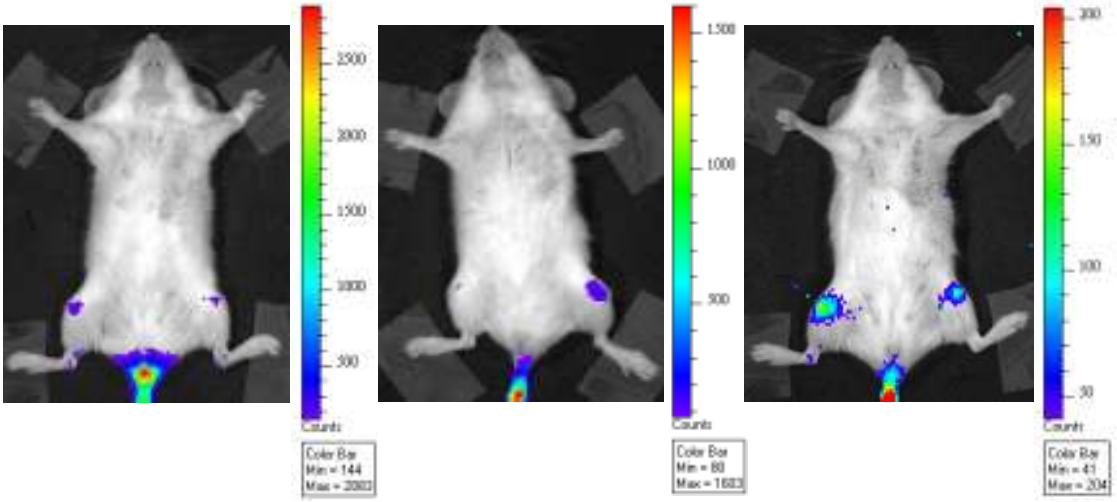


Figure 31. Firefly luciferase imaging in NOD/SCID mice, one week after injection with Molt-4 cells. NOD/SCID mice were injected through the tail vein with 20 million MOLT-4/GFP-Luc and cells were observed in the IVIS Lumina 1 week later (3 mg luciferin/mouse). A luminescent signal could already be detected in the femurs.

Despite the weak efficiency of *in vitro* transduction of Molt-4, we attempted an *in vivo* targeting experiment. For that, Sindbis/anti-FITC lentiviruses encoding DsRedIRESRenilla were produced in 100 mm culture plates and concentrated by ultracentrifugation. Four hundred ng (HIV p24) were incubated with anti-CD7-FITC (15 µg/mL) for 2 hours at room temperature and injected through the tail vein of each of the 2 engrafted mice, 2 weeks after cell injection. The third mouse was injected with virus only. The probability of success of this targeting experiment was expected to be very low, not only because of the difficulty in transducing this type of cells, but also due to the low amount of virus used. Morizono and collegues have reported *in vivo* targeting using 3 µg (HIV p24) of Sindbis-pseudotyped lentiviruses[152]. Nevertheless, it would be useful to test the renilla substrate coelenterazine. One week later (tree weeks after cell injection), this substrate was administered through retro-orbital injection (100 µg, directly from the supplied stock solution) and two mice died immediately. The third one was injected intraperitonially with the same coelenterazine and the image was acquired 15 minutes later. No renilla luminescence was detected, apart from the spot corresponding to site of the injection, indicating that no cell transduction occurred, despite the strong signal of the firefly luciferase detected in the femurs (Figure 32), that confirmed the good engraftment of Molt-4. Bone marrow cells were harvested from the femurs of the three mice and analysed by flow cytometry for GFP

(engraftment) and DsRed (tranduced cells) staining. The percentage of GFP was 20% and there was none for DsRed.

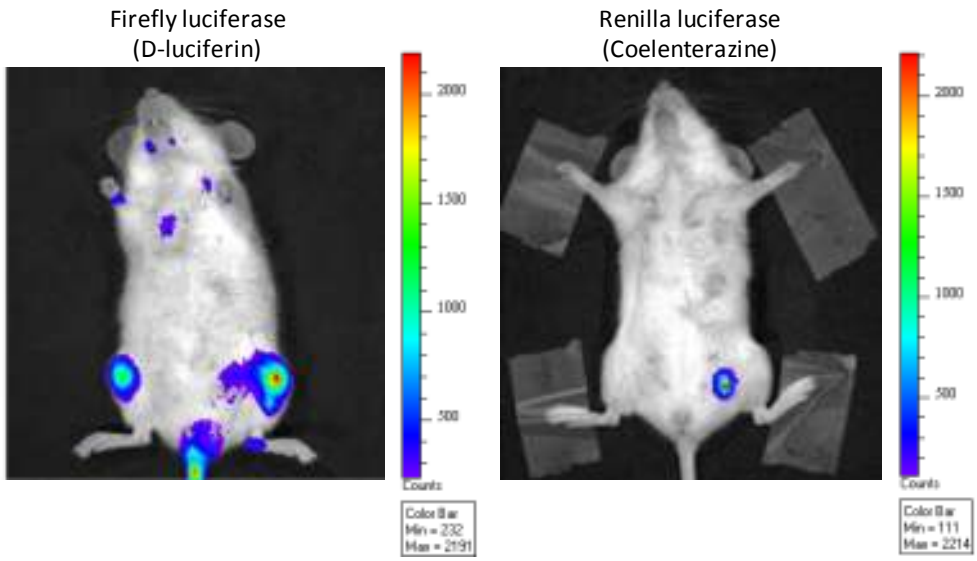


Figure 32. Luciferase imaging in NOD/SCID mouse three weeks after injection with Molt-4 cells. Mouse was injected through the tail vein with 20 million MOLT4/GFP-Luc and two weeks later injected through the same route with 400 ng (HIV p24) of Sindbis/anti-FITC M1234 lentiviruses encoding DsRedIRESRenilla, plus 15 µg/ml anti-CD7-FITC. Mouse was imaged in the IVIS Lumina one week later (3 weeks after cells injection). On the left is the image obtained after i.p. injection with 3 mg of D-luciferin and on the right is the image obtained after i.p. injection with 100 µg of coelenterazine.

To discard any problems with the renilla substrate coelenterazine, one Balb/c mouse was injected intraperitonially with 1.44 µg (HIV p24) of VSV/DsRedIRESRenilla lentiviral vector and imaged for renilla luciferase 4 days later (Figure 33). A good expression of renilla was observed in several organs beside the strong signal corresponding to the site of substrate injection. This indicated that this coelenterazine solution is good for i.p. injection, as the mouse did not die, and allows detection of transduced cells. However, this site of injection is not ideal as the luminescence associated with it can interfere with the detection of virus distribution (transduced cells).

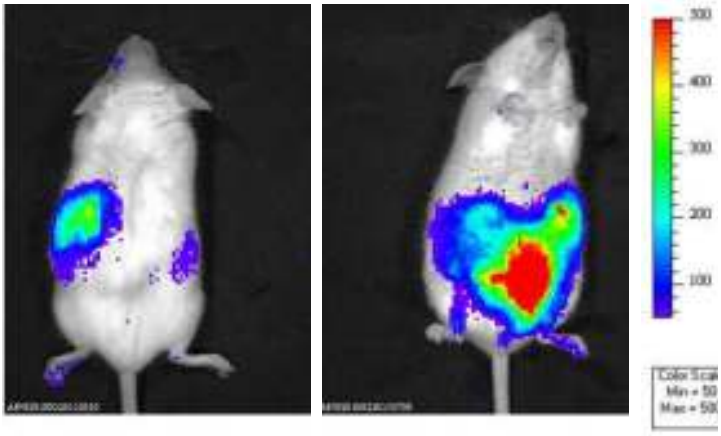


Figure 33. Renilla luciferase imaging in a Balb/c mouse. Mouse was injected intraperitonially with 1.44 µg HIV p24 VSV/DsRedIRESRenilla virus and imaged under the IVIS Lumina 4 days later (100 µg coelenterazine injected). Images were acquired 15 min after injection. Scale bar indicates luciferase counts.

Back to the issue of Jurkat cells engraftment, we decided to try the injection of new Jurkat cells in another strain, as the other Jurkat/GFP-Fluc were causing the death of mice (probably because they were contaminated with mycoplasm) and did not show any ability for engraftment. However, the engraftment of Jurkat cells in NOD/SCID mice has been described. NOD/SCID mice injected with 20×10^6 Jurkat cells showed 25% of human $CD45^+$ cells in the blood of 83% of mice, 10 days after injection[169]. High V1302 Jurkat cells engraftment levels (15×10^6 cells injected i.p.) were also achieved in irradiated NOD/SCID IL2R$\gamma^{-/-}$ mice (30% engraftment in the spleen and 11% in the bone marrow of 75% of transplanted mice) [7]. The other strain of mice that we had available at the time was the Rag2 $^{-/-}$ IL2Rγc $^{-/-}$. Thus, mice were injected with both the new and the old Jurkat cells. As those cells have no reporter gene, engraftment would have to be detected by staining mouse cells with an antibody for a human leukemic cell marker and further analysed by flow cytometry. Analysis of a sample of blood collected from mice would be enough to get an idea of the engraftment without the need to sacrifice them, as leukaemia cells are considered to have successfully engrafted if the proportion of human $CD45^+$ cells in the murine peripheral blood reach 1%[158]. Four weeks after injection, mice were sacrificed and bone marrow, spleen and blood were removed, stained with CD45-FITC antibody and analysed by flow cytometry (Figure 34). In the mice injected with the new Jurkat, there were human cells detected in all tissues analysed (except for the spleen of the two male

mice) but the highest percentage of engraftment was in the bone marrow. The old Jurkat cells were not able to engraft in this strain as well (data not shown).

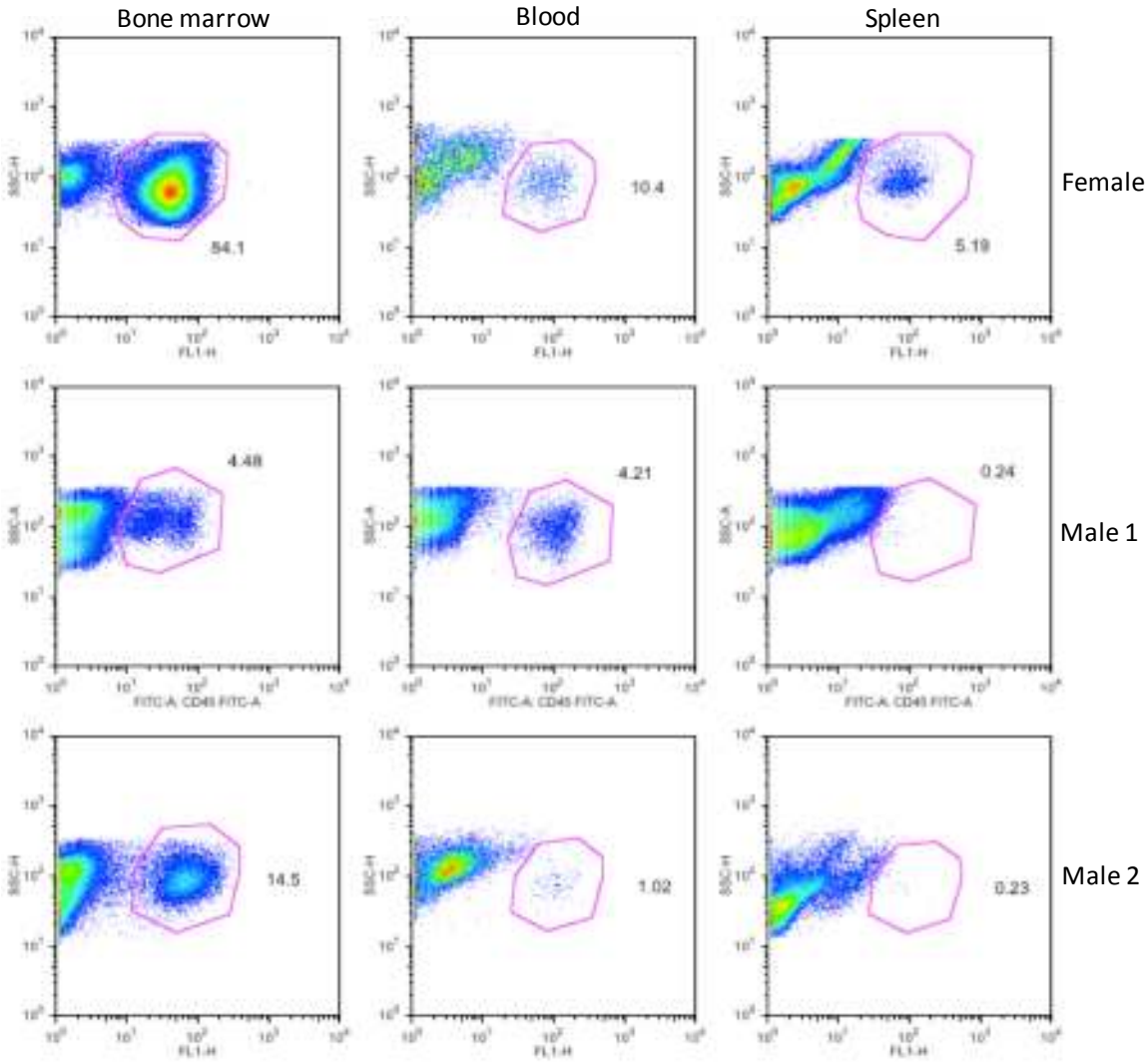


Figure 34. Percentage of human cells in Rag2 -/- IL2Rγc -/- mice injected with Jurkat. Three Rag2 $^{-/-}$ IL2Rγc $^{-/-}$ mice were injected by the tail vein with 20 million Jurkat cells. Four weeks later, they were sacrificed and the collected organs were stained with anti-CD45-FITC antibody and analysed by flow cytometry.

Given that the new Jurkat could be engrafted in the Rag2 $^{-/-}$ IL2Rγc $^{-/-}$, the cells were then transduced with VSV/GFP-Fluc lentiviruses, sorted by FACS and expanded. The expression of firefly luciferase from these cells was confirmed in a tissue culture plate analysed under the CCD camera (Figure 35). Subsequently, fifteen million cells were injected into two NOD/SCID mice. Four weeks later mice were imaged on IVIS

Lumina for firefly luciferase expression but there was no signal of cell engraftment (Figure 36).

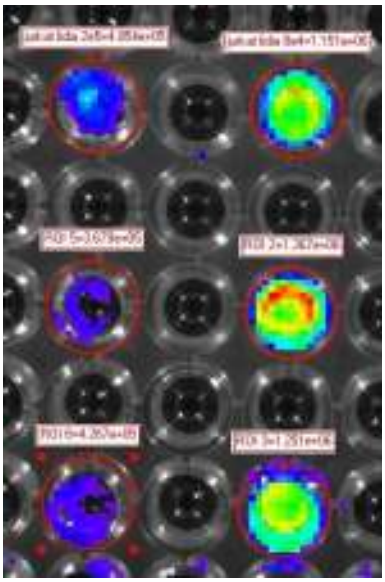


Figure 35. Imaging Jurkat/GFP-Fluc cells fluorescence in a culture plate. Eighty-thousand Jurkat/GFP-Fluc cells were plated on a 96-well plate in triplicate (right side). Jurkat cells (2×10^5) were included on the left side as a negative control. Fluorescence was detected in a CCD camera.

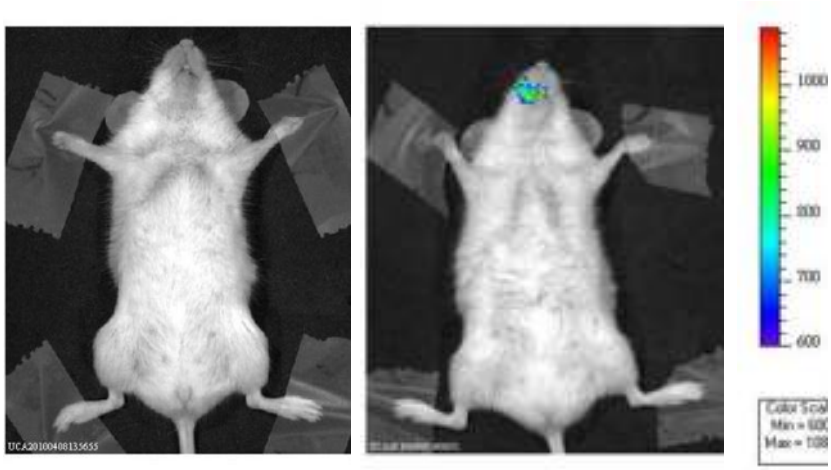


Figure 36. Firefly luciferase imaging of tumour cells distribution in NOD/SCID mice. Two NOD/SCID mice were injected by the tail vein with 15 million Jurkat/GFP-Luc and imaged 4 weeks later in the IVIS Lumina (3 mg luciferin/mouse). Scale bar indicates luciferase counts.

These results indicated that the Rag2 $^{-/-}$ IL2Rγc $^{-/-}$ was the best strain to proceed with the *in vivo* targeting experiments. Since it was not possible to obtain more of these mice, a similar one was purchased, the NOD-Rag1$^{-/-}$IL2rγ $^{-/-}$, a radioresistant strain that

support higher levels of engraftment than the parental NOD-SCID[164], as irradiation before cell injection facilitates engraftment.

Twenty million Jurkat/GFP-Fluc (98% GFP^+) cells were injected into 5 irradiated NOD-$Rag1^{-/-}$IL2rγ $^{-/-}$ mice and $10x10^6$ Jurkat/DsRedIRESRenilla (94% $DsRed^+$) were injected in another two irradiated mice. These ones were used for tests with coelenterazine (working solution, route of administration and time after injection). A working solution, diluted in PBS (containing only 5 mM of NaCl) immediately before retro-orbital injection, gave the best result and was less toxic to mice. A peak in renilla luminescence was observed when imaging 35 seconds after injection [1](data not shown). Mice injected with Jurkat/GFP-Fluc were analyzed in the IVIS Lumina for firefly luciferase expression one week later and all had a good engraftment (Figure 37), which was even higher than the one observed for NOD/SCID injected with Molt-4/GFP-Fluc, detected after the same period (Figure 31). Therefore, for the next experiments, our *in vivo* model of T-ALL will be based on the engraftment of Jurkat/GFP-Luc cells in irradiated NOD-$Rag1^{-/-}$IL2rγ $^{-/-}$ mice.

[1] Due to a bad reaction observed in some mice in subsequent experiments, which needed resuscitation procedures, we chose to perform image acquisition 1 minute after coelenterazine injection for all the animals in analysis.

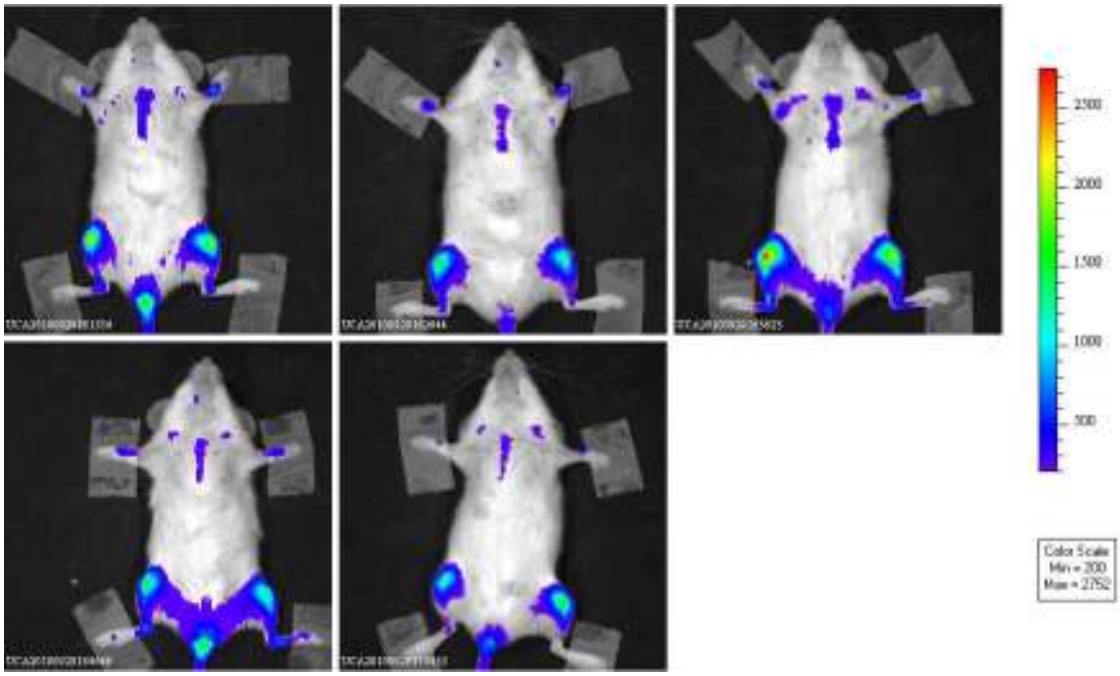


Figure 37. Firefly luciferase imaging in NOD-Rag1$^{-/-}$IL2rγ$^{-/-}$ mice one week after injection with Molt-4 cells. Mice were injected through the tail vein with 20 million Jurkat/GFP-Luc and cells were observed in the IVIS lumina 1 week later (3 mg luciferin/mouse). A strong luminescent signal can already be detected in the femurs. Scale bar indicates luciferase counts.

3.2.3 In vivo targeting of leukemic cells

Two weeks after Jurkat/GFP-Fluc injection in the first NOD-Rag1$^{-/-}$IL2rγ $^{-/-}$ mice tested for engraftment, 700 ng of Sindbis/anti-FITC M1234 expressing DsRedIRESRenilla were administered to each of the five mice. The lentiviruses (from a total amount of 3.6 µg) were obtained from transfection of 293T cells in 100 mm culture plates. Three mice were injected with virus plus 15 µg/ml anti-CD7-FITC and two were injected with virus only. Renilla expression was analysed 3 days and 7 days later but no luminescence signal was detected on the CCD camera (data not shown). This is probably related to the low amount of virus injected. As already mentioned, the total virus produced (3.6 µg) should be the quantity used for one mouse. This meant that viral production had to be scaled-up for *in vivo* use if we wanted to observe some targeting. Besides that, the fact that viruses are being injected some time after the injection of the cells may be influencing the result.

Another experiment was initiated, in which two irradiated NOD-Rag1$^{-/-}$IL2rγ $^{-/-}$ mice were injected through the tail vein with 20 million Jurkat/GFP-Fluc cells. Lentiviruses were produced in 150 mm tissue culture plates by a new protocol, concentrated by Lenti-X concentrator, followed by ultracentrifugation. Three days after cell injection, approximately 3 µg Sindbis/anti-FITC pseudotyped lentiviruses plus anti-CD7-FITC (15 µg/ml) and 1.5 µg VSV-G pseudotyped lentiviruses (used as a control), both expressing DsRedIRESRenilla, were injected by the same route. Mice imaging for detection of virus and cell distribution was performed 4 days after virus injection, which corresponds to one week after cell injection. There was co-localization of transduced cells expressing renilla luciferase (top of panel A, Figure 38) with the engrafted cells expressing firefly luciferase (top of panel B, Figure 38) in the femurs and the sternum (bone marrow), indicating that there was specific transduction of engrafted cells. Three days later, another imaging of these mice was performed and the signal from both luciferases was increased (bottom of each panel, Figure 38). The pictures on the left correspond to the mouse injected with VSV where it is also detected a signal in the spleen, besides the one observed in the bone marrow. We were expecting more background transduction for the mouse injected with VSV pseudotyped lentiviruses considering its broad tropism and the results obtained by

Morizono and colleagues[152], where it was also reported transduction of the liver and lungs. Curiously, this mouse had not only a higher level of transduction but also a higher level of cell engraftment.

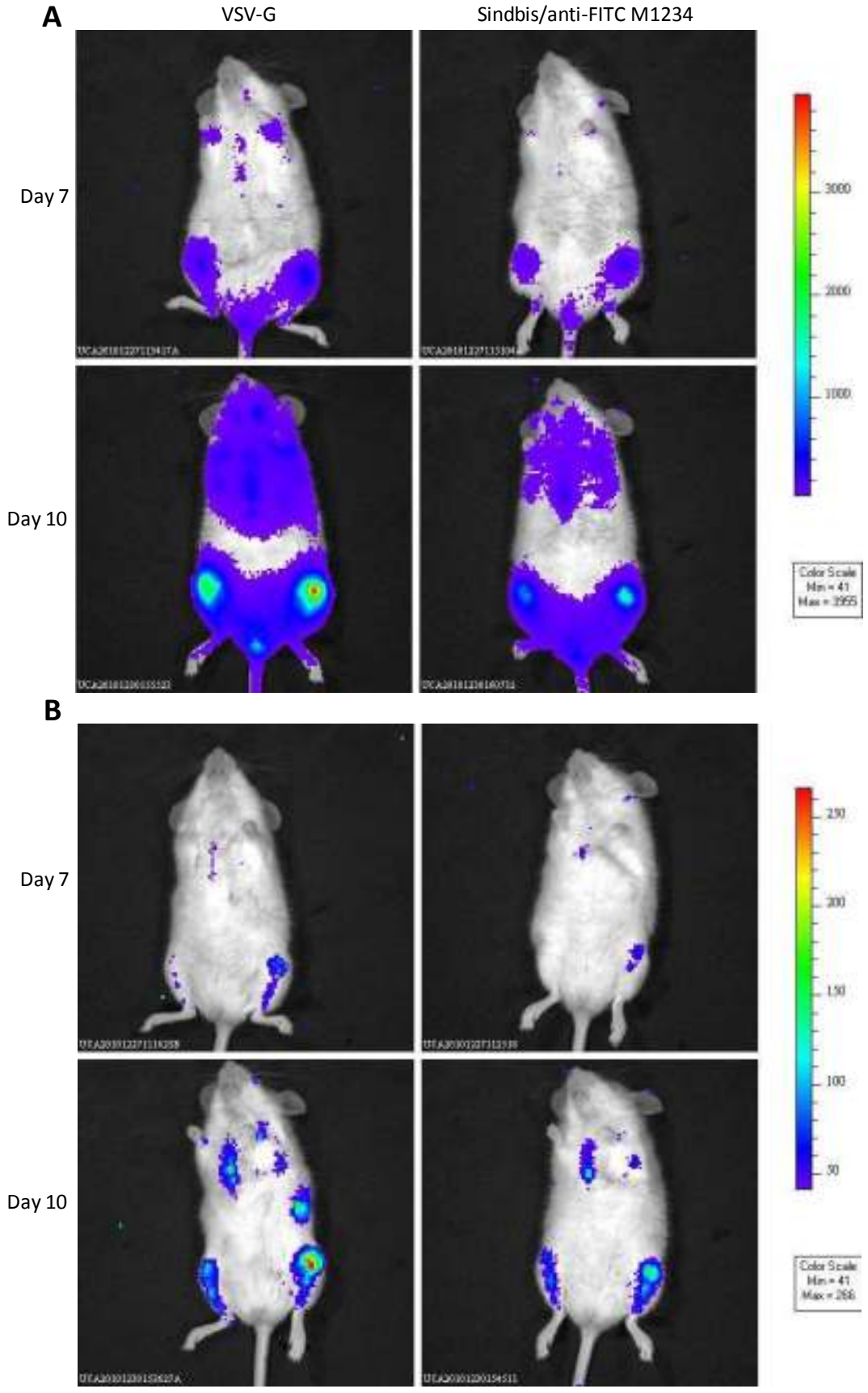

A
VSV-G
Sindbis/anti-FITC M1234
Day 7
Day 10
3000
2000
1000
Color Scale
Min = 41
Max = 3955
B
Day 7
Day 10
250
200
150
100
50
Color Scale
Min = 41
Max = 266

Figure 38. Sindbis/anti-FITC M1234 pseudotyped lentiviral vector can target specific leukemic cells after systemic delivery in mice. Three days after cell injection (20 million *per* mouse), NOD-Rag1$^{-/-}$IL2rγ$^{-/-}$ mice were injected through the tail vein with VSV-G pseudotyped lentiviruses alone (left pictures) or Sindbis/anti-FITC pseudotyped lentiviruses plus 15 µg/mL anti-CD7-FITC antibody (pictures on the right), both expressing DsRedIRESRenilla. Four days later, the level of engraftment was determined by imaging the expression of firefly luciferase (top of A) and virus infection was determined by imaging the expression of renilla luciferase (top of B). On the bottom of each of the panels A and B are the images acquired three days later (day 10). Scale bars indicate luciferase counts.

These results indicated that the Sindbis/anti-FITC M1234 lentiviruses were able to target the leukemic cells *in vivo*. In order to have an idea of the percentage of transduction within that cell population it was necessary to perform a flow cytometry analysis of the bone marrow cells and for that, it was essential to have a control mouse injected only with cells. Therefore, Jurkat/GFP-Luc cells were injected in two irradiated NOD-Rag1$^{-/-}$IL2rγ $^{-/-}$ mice as described before and three days later Sindbis/anti-FITC M1234 lentiviruses plus anti-CD7-FITC were injected in one mouse. Seven days after cell injection, mice were imaged on the IVIS Lumina to verify the engraftment, which was considerably lower in the mouse that was given the virus (Figure 39A). The renilla signal (transduced cells) (Figure 39B) was much weaker than the previously obtained (Figure 38), even at day 10. Nevertheless, the femurs were removed and bone marrow cells were collected for flow cytometry analysis. According to Figure 40, the engraftment was more than10 times lower for the mouse injected with virus than for the mouse injected only with cells (2% versus 22% of GFP$^+$ cells, respectively), as was expected from the images obtained in the CCD camera. Within this engrafted population, there were 2.58 % of DsRed$^+$ cells (transduced cells).

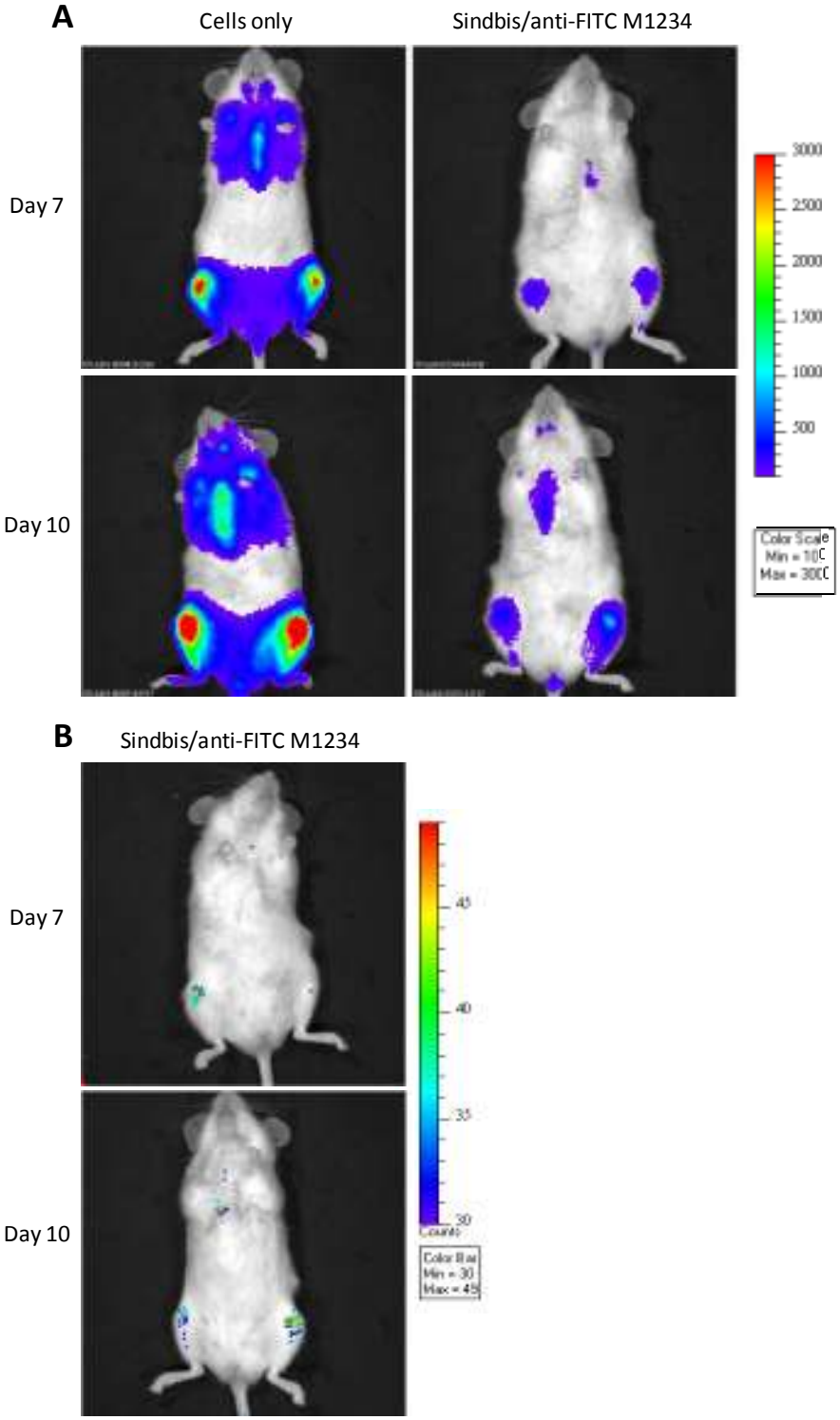
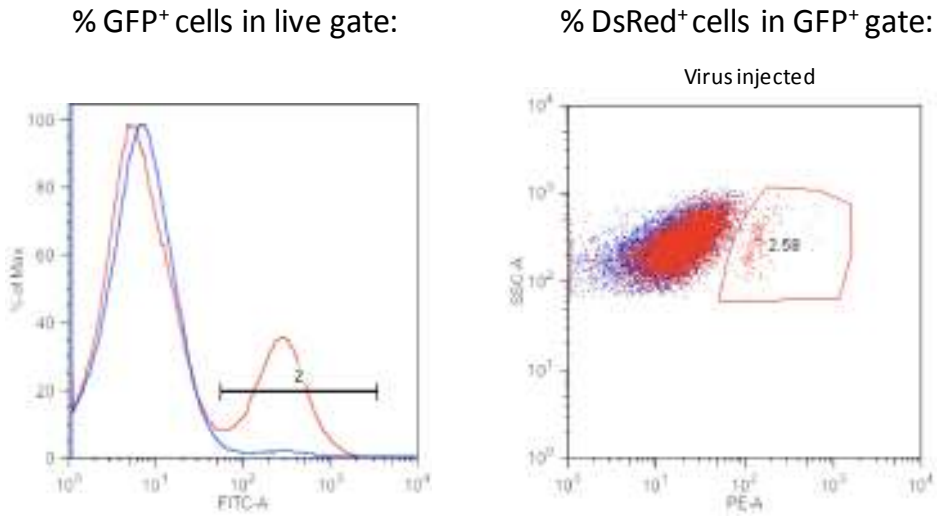


Figure 39. Sindbis/anti-FITC M1234 pseudotyped lentiviral vector can target specific leukemic cells after systemic delivery in mice. Three days after cell injection (20 million *per* mouse) in two NOD-Rag1$^{-/-}$IL2rγ$^{-/-}$ mice, one of them was injected through the tail vein with Sindbis/anti-FITC pseudotyped lentiviruses expressing DsRedIRESRenilla plus 15 µg/mL anti-CD7-FITC antibody while the other one was left untreated. Four days later, the level of engraftment was determined by imaging the expression of firefly luciferase (top of panel A) and virus infection was determined by imaging the expression of renilla luciferase (top of panel B). On the bottom of each of the panels A and B are the respective images acquired three days later (day 10). Scale bars indicate luciferase counts.



Cells only	22%
Virus injected	2%

Figure 40. Efficiency of *in vivo* transduction of leukemic T cells with Sindbis/anti-FITC M1234 pseudotyped lentiviral vector. The femurs from two mice, the one injected with Sindbis/anti-FITC M1234 lentiviral vector and the non-injected one (cells only), were removed and bone marrow cells were analysed for engraftment (GFP^+) and transduction efficiency (GFP^+ $DsRed^+$) by flow cytometry.

The data so far indicated that Sindbis/anti-FITC M1234 lentiviruses were able to transduce the leukemic T cells when injected 3 days after cell administration. To check if the viruses would have a better ability to target the cells if injected earlier on, i.e. before tumour cells started to expand, an experiment was performed in which the virus was injected on the day following cell injection. As it can be observed in Figure 41, tumour cells were also transduced in this way. Although the intensity of both luciferase signals are lower than those obtained on a previous experiment (Figure 38), the ratio between engrafted and transduced cells is not so marked, which might indicate a better efficiency of transduction. To quantify that efficiency by flow cytometry, the femurs were removed and bone marrow cells were analysed for GFP^+ (tumour cells) and within this population were gated for $DsRed^+$ (transduced cells). The percentage of transduced cells was 15.2% within an engrafted population of 0.22% (Figure 42). This was almost 10 times lower than previous data indicating 2% engrafted cells. Actually, this is in accordance with the IVIS Lumina data, as the signal intensity for firefly luciferase in this experiment is almost 10 times lower than the previous one (Figure 38).

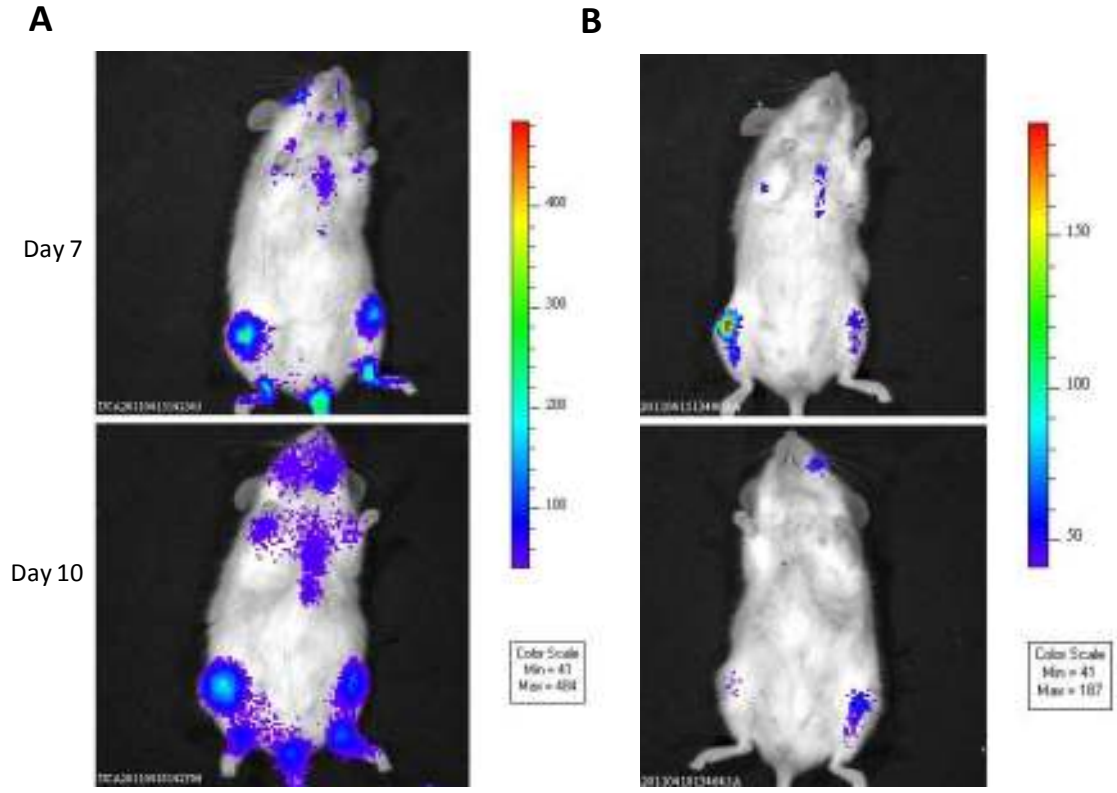


Figure 41. Sindbis/anti-FITC M1234 lentiviral vector can target specific leukemic cells after systemic delivery in mice. One day after cell injection (20 million *per* mouse), one mouse was injected through the tail vein with Sindbis/anti-FITC plus 15 µg/mL anti-CD7-FITC antibody. Six days later, the level of engraftment was determined by imaging the expression of firefly luciferase (panel A) and virus infection was determined by imaging the expression of renilla luciferase (panel B). On the bottom of each of the panels A and B are the images acquired ten days after cell injection. Scale bars indicate luciferase counts.

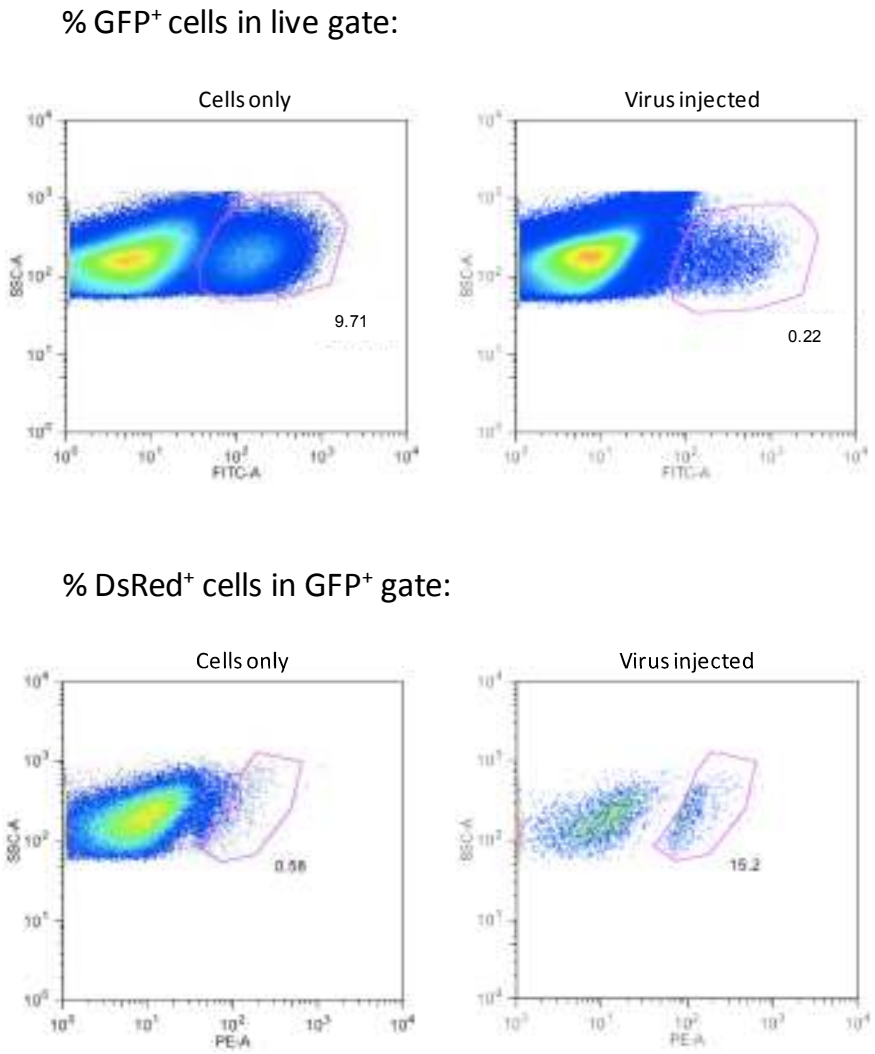


Figure 42. Efficiency of *in vivo* transduction of leukemic T cells with Sindbis/anti-FITC M1234 pseudotyped lentiviral vector. The femurs from the Sindbis/anti-FITC M1234 lentiviral vector injected mouse were removed and bone marrow cells were analysed for engraftment (GFP^+) and transduction efficiency (GFP^+ $DsRed^+$). On top are GFP gated cells and on bottom are $DsRed^+$ within that population.

This data demonstrated not only the *in vivo* capacity and efficiency of this Sindbis/anti-FITC targeting strategy but also gave an indication of the best timing for its application. The effectiveness of targeting tumour cells is dependent upon factors such as the ratio of vector to tumour cells and accessibility of tumour cells to the vector. Therefore, the number of vector particles should be adequate to infect a significant percentage of leukemic cells *in vivo*. As smaller tumours contain fewer cells, the same amount of virus should result in better rates of infection, and consequently greater tumour regression would be expected when an *in vivo* suicide strategy is applied. Therefore, the best option is to start the virus injection earlier on.

Although these *in vivo* targeting experiments were performed to demonstrate that we could achieve a significant targeting efficiency and to optimize conditions for the *in vivo* suicide gene therapy (next section), they should have been performed with more mice *per* each condition to have a statistically significant result. Different results observed between mice could be attributed to variability from mouse to mouse and differences in injection efficiency within the groups of animals (for each condition). However, the NOD-Rag1$^{-/-}$IL2rγ $^{-/-}$ mice were very expensive and this prevented us from carrying out these initial targeting experiments using a larger number of animals.

3.2.4 In vivo killing of leukemic cells

We have demonstrated that the Sindbis/anti-FITC M1234 lentiviruses encoding HSV-TK and DsRed (DsRedIRESTK) can kill the transduced leukemic cells *in vitro*. However, to show the efficiency of this suicide gene *in vivo,* that reporter gene would have to be substituted by renilla in order to monitor its luminescence signal after treatment with GCV. The lentiviral plasmid FUW/RenillaIRESTK was generated and its expression was confirmed in 293T transfected cells, both by alamarblue and by luciferase assay (Appendix E). Therefore, lentiviral vectors were produced and concentrated. Mice were injected with Jurkat/GFP-Fluc cells and on the following day, a group of 4 mice were injected with viruses (approximately 3 µg HIV p24 *per* each mouse) and a group of 8 were injected with same amount of viruses plus anti-CD7-FITC (15 µg/ml). The latter group would be further divided into two, one to which would be administered GCV (10 mg/Kg) and other that would be left untreated. Ten days after cell injection, mice were analysed on IVIS lumina for renilla (transduction) and firefly (engraftment) luciferase expression. As it is shown in Figure 43 (it includes only the 2 groups to which was given virus plus antibody), there was a good cell engraftment but no specific transduction was detected and consequently, GCV was not administered. A week later, mice were observed again but without any progress, i.e., they remained without signs of cell transduction, and therefore, they were sacrificed. If the transduction efficiency was at least 2 times the maximum value obtained previously (15.2%), we could observe the effect of GCV by monitoring the reduction of the engraftment (reduction in signal intensity of firefly luciferase), which would likely be even higher due to its known bystander effect.

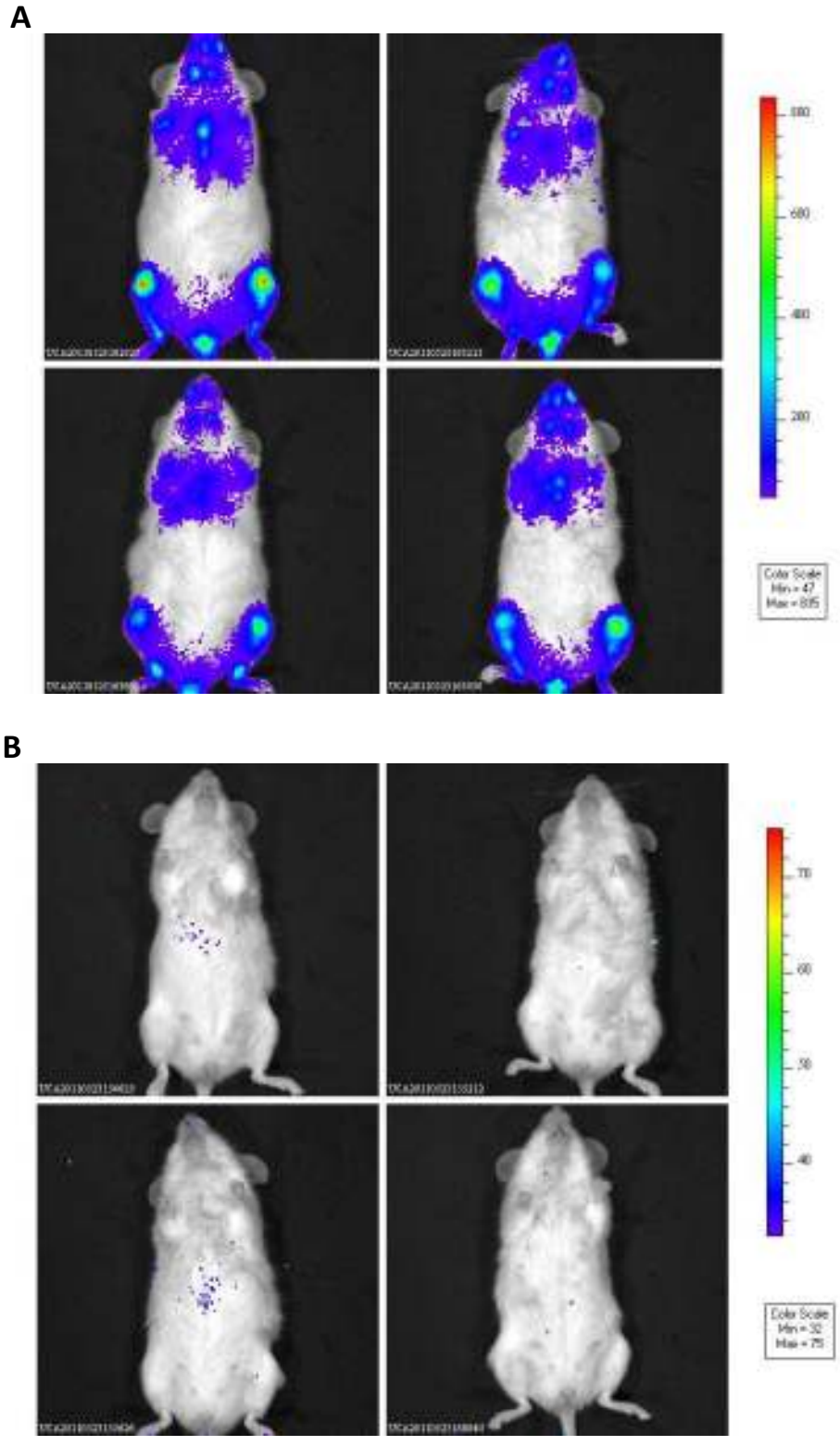


Figure 43. Sindbis/anti-FITC M1234 lentiviral vector expressing RenillaIRESTK was not able to target specific leukemic cells upon systemic delivery in mice. One day after cell injection (20 million *per* each mouse) mice were injected through the tail vein with Sindbis/anti-FITC RenillaIRESTK lentiviruses (3 µg/ml HIV p24 per mouse) incubated with anti-CD7-FITC (15 µg/ml). One week after cell injection, they were analysed on the IVIS lumina for firefly (A) and for renilla (B) expression. Scale bars indicate luciferase counts.

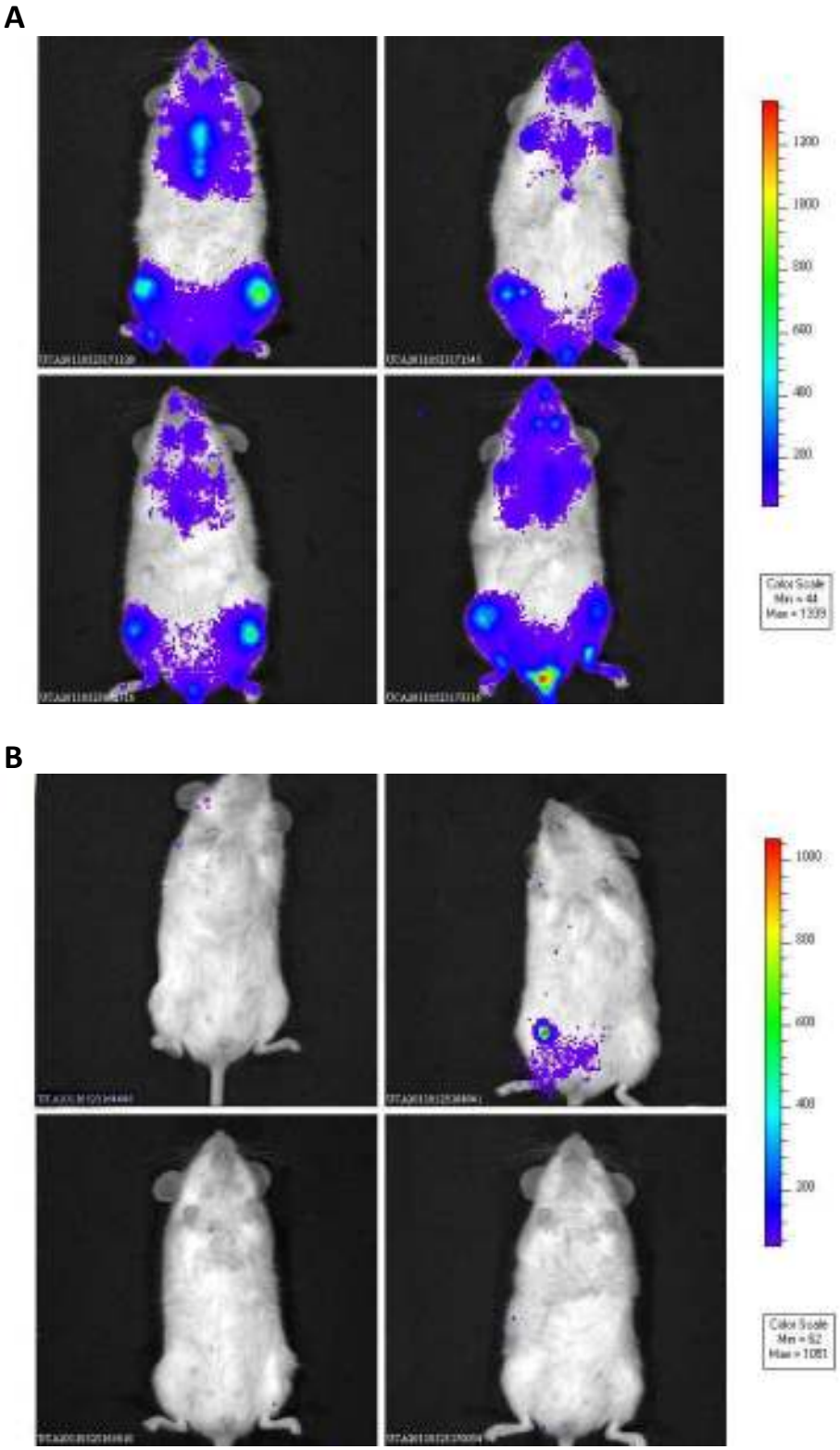


Figure 43. ***Continued (2nd group of mice).***

This result was not expected at all, as it was demonstrated in several experiments before that Sindbis/anti-FITC M1234 lentiviruses could target and deliver the

transgene. The problem would probably reside on the renilla expression from the FUW/RenillaIRESTK lentiviral plasmid. To compare the efficiency of transduction mediated by the two lentiviral vectors encoding RenillaIRESTK or DsRedIRESRenilla, Sindbis-anti-FITC M1234 lentiviruses with both constructs were produced in parallel and each one (plus labelling antibody) was injected in two mice, the day after cell injection. Fifteen days later, mice were analysed on the IVIS lumina for firefly and renilla expression. One of the mice injected with Sindbis/anti-FITC M1234 encoding DsRedIRESRenilla died during coelenterazine administration. As shown in Figure 44, it was detected expression of renilla from the mouse injected with virus expressing DsRedIRESRenilla but not in the mice injected with the other virus. Thus, mice were killed and femurs were removed. Bone marrow cells were harvested and genomic DNA was extracted and kept frozen for further analysis. Vector DNA integration gives an indication (quantitative) of the efficiency of transduction and can be more accurate than analysis of transgene expression in target cells or tissues that may be influenced by variability in vector expression levels[185]. To check if the viruses were able to integrate or not, it was performed a real-time PCR with primers to amplify renilla luciferase gene from the genomic DNA. Vector copy numbers are indicated on Table IX. The mice injected with the virus encoding RenillaIRESTK had unexpectedly higher vector copy numbers integrated on its genome (138 and 151 copies) than the mouse injected with the virus encoding DsRedIRESRenilla (33 copies). This demonstrates that both viruses could target the cells and indicate that there was a problem with the expression of renilla from the RenillaIRESTK virus. To confirm this, Jurkat cells were transduced with VSV-G pseudotyped lentiviruses encoding each of the constructs RenillaIRESTK and DsRedIRESRenilla. Three days later, cells were plated in a 96-well plate for analysis of renilla luciferase expression in the IVIS lumina. As expected, there was no luminescence signal from the Jurkat/RenillaIRESTK while there was a signal for the Jurkat/DsRedIRESRenilla (Figure 45). It was shown that an unusual very high expression of the IRES-driven second transgene may result in down regulation of expression of the first transgene, or exert a direct toxic effect on target cells[66]. That might have been what happened with our construct. The lentivirus that had integrated more copy numbers on the genomic DNA was the one that showed no renilla expression. The renilla expression levels from

the RenillaIRESTK construct was probably so weak that could not be detected in the IVIS lumina. However, the FUW/RenillaIRESTK plasmid expressed high levels of the first transgene in an *in vitro* assay in 293T cells (Figure 50, Appendix E) and this led us to consider that it would also express properly upon transduction in Jurkat cells.

Although it was shown on section 3.1.8.2 that the TK construct, which allowed the highest expression of the other transgene was the one where TK expression was mediated by an IRES, other constructs, with different positioning of the renilla and TK genes, should be cloned and its expression tested in transduced Jurkat cells in order to overcome this problem with the renilla expression.

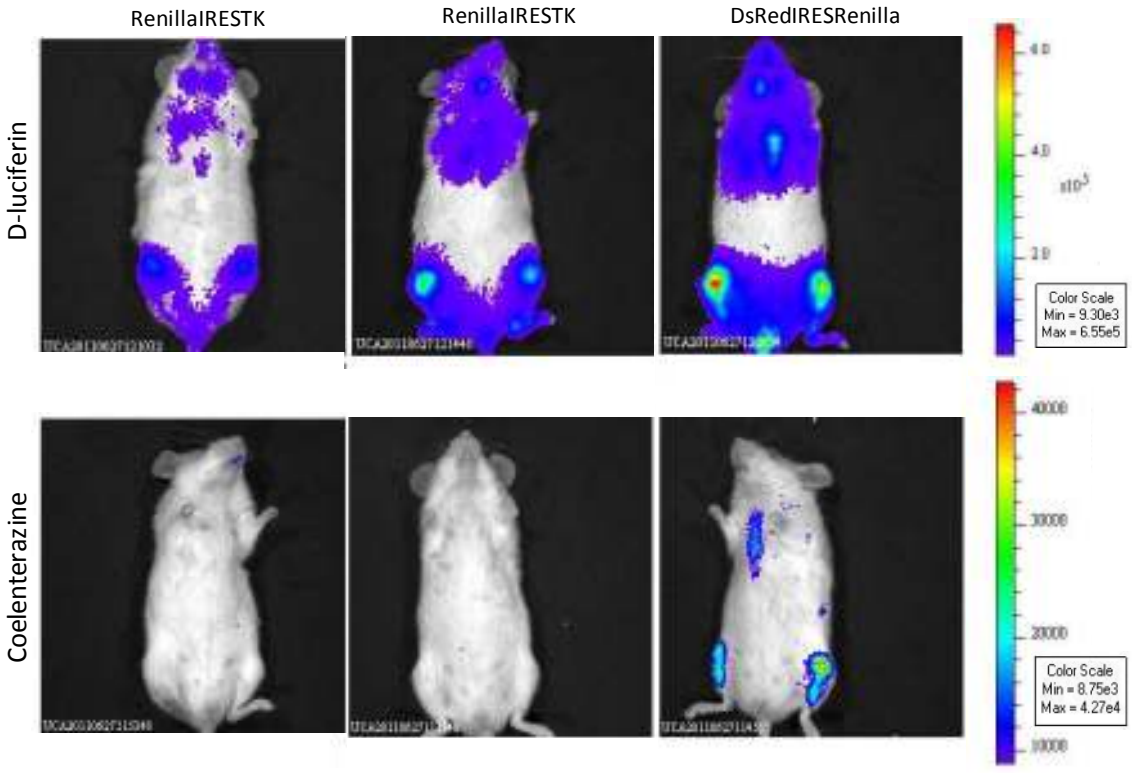


Figure 44. Comparison of the efficiency of *in vivo* targeting between Sindbis/anti-FITC pseudotyped lentiviral vectors expressing DsRedIRESRenilla or RenillaIRESTK. One day after cell injection (20 million *per* mouse) mice were injected through the tail vein with 3 µg/ml Sindbis/anti-FITC M1234 expressing DsRedIRESRenilla or RenillaIRESTK plus anti-CD7-FITC (15 µg/ml). 15 days later, mice were injected with coelenterazine for analysis of renilla expression (transduction, Bottom) and with D-luciferin for analysis of firefly expression (engraftment, Top). Scale bars indicate photonic flux (photons/sec/cm^2/steridian).

Table IX. Copy numbers of lentiviral vectors expressing DsRedIRESRenilla or RenillaIRESTK. Genomic DNA was extracted from bone marrow isolated from femurs of the mice indicated in Figure 44.

Mouse	Vector	Copy number/10^4 cells
2	DsRedIRESRenilla	33
3	RenillaIRESTK	138
4	RenillaIRESTK	151

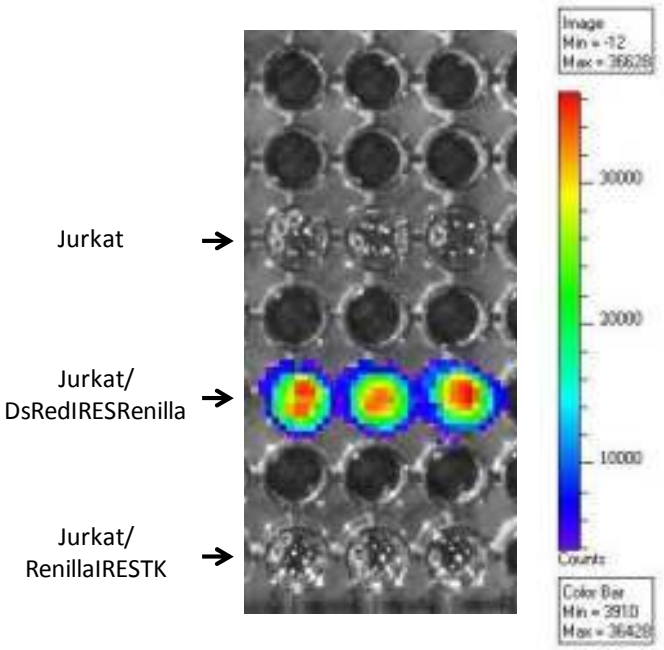


Figure 45. Comparison of the renilla luminescence from Jurkat cells expressing DsRedIRESRenilla or RenillaIRESTK. One hundred µl of Jurkat cells transduced with VSV-G/DsRedIRESRenilla or VSV-G/RenillaIRESTK lentiviruses were plated in triplicate on a 96-well plate and 100 µl of a 20µM coelenterazine solution was added to each well. Untransduced Jurkat cells were included as a negative control. Renilla luminescence was immediately analysed in the IVIS lumina (exposure time: 1 min, binning large, f/stop 1).

4. Conclusions and perspectives

For *in vivo* gene therapy applications, it is very important that the method of delivery would target only specific cells or tissues while sparing the surrounding ones. This is essential not only to provide an efficient gene therapy application, but also to enhance its safety, as inadvertent infection of irrelevant cells or tissues can result in serious adverse effects in clinical trials [112]. Targeting of the viral vector can be accomplished most often by modifying the envelope structure through the display of recognition sequences on the surface or applying tissue specific promoters. The insertion of a ligand or a scFv with higher affinity for target molecules increases the affinity of the envelope protein and results in higher transduction efficiency. Since specificity is advantageous for *in vivo* gene therapy strategies, it is preferable to use antibodies as targeting strategy given the specificity with which they recognize their targets. In this study, we used an anti-FITC scFv incorporated on the Sindbis virus envelope for specific targeting of FITC-labelled cells. This is the first time that an anti-FITC scFv is used as a targeting strategy for gene therapy. It could be easily displayed at the surface of the Sindbis viral envelope and the lentiviruses can have a relatively high titer. Mutations in the envelope glycoprotein were further introduced to reduce *in vivo* non-specific transduction, as shown by Morizono and colleagues[152], although it slightly reduced the viral titer comparing to wt (without mutations).

This strategy of targeting an organic molecule with a scFv has advantages over others, such as redirecting vectors through antibody conjugation, which is a problem in immunocompetent animals due to competition with plasma antibodies. Moreover, it avoids cloning of new scFv whenever one wants to target a new cell receptor, as there are FITC-conjugated antibodies for many receptor molecules.

We have shown that an HIV-derived lentiviral vector pseudotyped with this Sindbis/anti-FITC specifically infects T cells that are labelled with a CD7 FITC-conjugated antibody, but not CD19 labelled cells, that is a B cell receptor. The lentiviral vector Sindbis/ST6 that displays a scFv recognizing other cell receptor (CCR5) could not transduce the same T cells. The highest specific targeting efficiency of Jurkat/CD7-FITC cells reached was 82.8% for Sindbis/anti-FITC Wt lentiviruses (Figure 19A) and 44.5% for Sindbis/anti-FITC M1234 lentiviruses (Appendix C), both

expressing a DsRed reporter. Furthermore, the lentiviruses binding ability for Jurkat/CD7-FITC cells could be reduced by competition with an anti-FITC soluble antibody, demonstrating that the targeting is specifically mediated by the scFv display and the need of binding to FITC for viral entry. These results altogether demonstrate that the Sindbis envelope pseudotyped lentiviral vectors containing anti-FITC scFv specifically and preferentially infect cells displaying a FITC-conjugated molecule.

A HSV-TK/GCV based suicide gene strategy was employed to specifically kill the leukemic T-ALL cells. Previously, Dr. Meruelo group has demonstrated the potential of this kind of suicide gene therapy for targeting using a cell type-specific recombinant Sindbis virus vector and an antibody system[87]. Others have demonstrated specific targeting and induction of apoptosis of T-ALL cells using a scFv against human CD7 fused to an immunotoxin, more precisely the catalytic domain of Pseudomonas exotoxin A fragment[165]. Using our Sindbis/anti-FITC pseudotyped lentiviral vector system we were able to target leukemic Jurkat cells and deliver the HSV-TK gene that, in the presence of 1 μg/mL GCV, lead to a gradual reduction in the percentage of transduced cells (Figure 26). In fact, using this HSV-TK/GCV suicide gene strategy, we were able to kill 95% of the transduced cells. However, i*n vivo*, and in a clinical setting, the inability to totally kill the tumour cells for any strategy considered alone indicates the need for combined therapies in order to achieve a satisfactory treatment of the tumour.

As an *in vivo* proof of concept of this strategy, we used an animal model of T-ALL. It was reported that childhood ALL cells retain the phenotypic and genotypic characteristics of the original patient sample after being engrafted in NOD/SCID mice[20]. In our model system, the tumour was generated by intravenous injection of Jurkat T-ALL cells into immunodeficient NOD.Rag1$^{-/-}$γc$^{-/-}$ mice and it could be detected one week later. Using Sindbis/anti-FITC M1234 expressing DsRedIRESRenilla we were able to transduce 15.2% of the tumour cells. An inefficient production and purification of the lentiviral vector may account for the relatively low efficiency of the *in vivo* transduction. Hence, to improve the *in vivo* efficiency of our gene therapy strategy, it would be better to use clinical-grade vector

manufacturing. Moreover, it should be considered the further scaling-up of vector production, replacing the time-consuming and labour-intensive small-scale vector production in cell culture dishes.

To test whether our system could deliver the suicide gene HSV-TK *in vivo*, Sindbis-pseudotyped lentiviruses encoding RenillaIRESTK were injected in mice on the day following cell injection (thus, before the tumour established). Even if only a small fraction of the tumour cells were transduced and expressing the HSV-TK gene it would be observed a significant reduction in the tumour size after GCV treatment due to its bystander effect. Nevertheless, we could always re-administer another dose of the virus to enhance the efficiency of this gene therapy. However, due to a problem with the expression of the renilla reporter gene in our lentiviral system, that prevented the detection of the transduced cells in the IVIS lumina, the treatment with GCV was not initiated.

Taken together, these results indicate that our gene therapy system is suitable for systemic delivery and can be applied for disseminated diseases and other disease settings for which there is an available surface receptor or ligand FITC-labelled antibody. For instance, it can be applied to any cancer cell, which normally have certain receptors overexpressed and to which there are antibodies available, such as the case of epidermal growth factor receptor (EGFR) that is commonly overexpressed in several tumour cell lines. The availability of other cell-specific surface molecules conjugated with FITC will broaden its application in therapy and research.

As already mentioned, for a successful gene therapy it is important to achieve a high efficiency of transduction to the desired cell types, which will reduce the dose needed for effective therapy, thereby reducing undesirable side effects. Hence, even though the strategy of gene therapy here proposed has great potential for future applications, it could be further improved, particularly the delivery of the scFv. Other means could be used to deliver this scFv. For instance, it could be chemically coupled to liposomes, nanoparticles, or fused to cationic polymers such as protamine. However, although non-viral vectors should probably be less toxic to the cells, they might not be as

efficient as viral vectors (lentiviral vectors in this case) to deliver the scFv. Other approach would be to substitute the Sindbis viral envelope by another one. Pseudotyping by measles virus (H and F proteins) have emerged as alternative approaches for lentiviral vector targeting[77, 78, 6] and have been also shown to incorporate a scFv and display it at the viral surface. As previously mentioned, Sindbis entry relies on the low pH within the endosomes to mediate fusion to achieve targeted transduction. The direct fusion entry mechanism of measles virus has the advantage of avoiding the step of endosome escape. Nevertheless, in an immunocompetent host, an endosomal entry could potentially protect viruses from the host immune system. Measles and Sindbis virus envelopes have the binding and fusion functions separated into different glycoproteins, and therefore are a good choice for scFv or other ligand incorporation for retargeting as it can be generated binding-deficient but fusion competent mutants[152, 77]. In a study by Funke and colleagues[77], they targeted the cell surface molecules EGFR and CD20 through display of the EGF ligand or a scFv as a C-terminal extension of the H measles virus glycoprotein in a lentiviral vector cell entry targeting system. They report lower background transduction on control cells than that in the study by Yang and colleagues [240] also targeting CD20 but using a Sindbis-pseudotyped lentiviral vector. The Sindbis glycoproteins are restricted to target cell surface molecules that need to undergo rapid endocytosis. More recently, they targeted different cell surface molecules on different cell types taking advantage of scFv for those molecules and where natural receptor usage is prevented by mutation of the relevant residues in the HA protein receptor recognition domain. However, for future clinical applications in humans the therapeutic efficacy may be reduced due to pre-existing Measles virus neutralizing antibodies. Exchange of envelope glycoproteins of the oncolytic virus with those of the closely related canine distemper virus (CDV) could allow viral escape and achieve *in vivo* targeted therapy[142]. Nevertheless, measles virus pseudotyped lentiviral vectors have not been applied so far for systemic delivery *in vivo* in animal models.

Another aspect that could be further improved in our model system is to increase avidity therefore, enhancing binding to target cells, using for instance a bi-specific diabody or even a higher multivalent scFv. An enhanced avidity has been shown to be

beneficial for in vivo tumour targeting in many antibody-based therapeutic strategies [106].

The work presented here, including the optimizations for viral production, cell transduction and suicide gene strategy, will have important implications for future projects in the field of gene therapy, in particular those being developed at this laboratory.

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Appendices

Appendix A – list of primers

Table X. List of the primers used for sequencing.

Name	Purpose	Primer sequence (5'→ 3')
5'-FITC	5'anti-FITC sequencing primer (Reverse direction)	AGA TAC CAC CTC AGG TAG
4MFwd	3' anti-FITC sequencing primer (Forward direction)	AGC GTG TAC CTG CAG ATG
UbiFwd	FUW forward primer	TCA GTG TTA GAC TA G TAA ATT G
E2.end (S)	Sindbis E2 sequencing	TGG CCC CAA ACG CCG TAA TCC
E1.end (AS)	Sindbis E1 sequencing	TTC GTG TGC TAG TCA GCA TCA TG

Table XI. List of the primers used for Sindbis envelope mutagenesis. It includes the purpose (which mutant) and the primer sequence. The template was Sindbis/anti-FITC Wt. In bold are the mutation sites.

Name	Purpose (Mutant)	Primer sequence (5'→ 3')
159KE160AA.S	159KE160AA (M3)	CAGTGTACGACCGTCTG**GCAGCA**ACAACTG CAGGCTACATC
159KE160AA.AS	159KE160AA (M3)	GATGTAGCCTGCAGTTGT**TGCTGC**CAGACG GTCGTACACTG
SLKQ68-71AAAA.S	SLKQ68-71AAAA (M2)	CAAGTACCGCTACATG**GCGGCTGCGGCG**G TAACCGGAGGCGG
SLKQ68-71AAAA.AS	SLKQ68-71AAAA (M2)	CCGCCTCCGGTTAC**CGCCGCAGCCGC**CATG TAGCGGTACTTG
delE362-64.S	delE362-64 (M1)	GCGGTGCGGATCGTCT**GGCAGC**GTCATTGA CGACTTTACC
delE362-64.AS	delE362-64 (M1)	GGTAAAGTCGTCAATGAC**GCTGCC**AGACGA TCCGCACCGC
226AK227SG.S	226AK227SG (M4)	TAGGCTACTCAAGCCTTCC**AGCGGG**AACGT GCATGTCCCGTAC
226AK227SG.AS	Mutant 226AK227SG (M4)	GTACGGGACATGCACGTT**CCCGCT**GGAAGG CTTGAGTAGCCTA

Table XII. List of the primers used for cloning. It includes the name of the construct and the gene being amplified, the primer sequence and the template. Underlined are the START, or an in frame start, and STOP codons; in bold are the restriction sites.

Name	Name of construct (gene amplified)	Primer sequence (5'→ 3')	Template
DsRed.NheI (S)	DsRed-Vpr (DsRed)	AT**GCTAGC**CACCATGGATAGCAC TGAG	pIRES2DsRed -Express2
DsRed.HindIII (AS)	DsRed-Vpr (DsRed)	C**AAGCTT**CTGGAACAGGTGGTGG C	pIRES2DsRed -Express2
Vpr (S)	DsRed-Vpr (Vpr)	CCTGTTCCAGAAGCTTGAACAAGC CCCAGAAG	pEGFP-Vpr
Vpr. XbaI (AS)	DsRed-Vpr (Vpr)	GTGGATCC**TCTAGA**CTAGGATCT ACTGGCTCC	pEGFP-Vpr
RFP.BamHI (S)	FUW-RFP	**GGATCC**GTCGCACACCATGGCCT	#318/RFP
RFP.EcoRI (AS)	FUW-RFP	A**GAATTC**GAAGCTTGAGCTCGAG ATC	#318/RFP
Luc. BamHI (S)	FUW-Luc	T**GGATCC**ACCATGGAAGACGCCA AA	pGL3-Basic
Luc. EcoRI (AS)	FUW-Luc	CGC**GAATTC**TCTAGAATTACACG GCGATC	pGL3-Basic
IRES2-DsRed (S)	FUW-IRESDsRed	TCGAATTCTGCAGTCGACGGTACC	pIRES2DsRed -Express2
IRES2-DsRed.EcoRI (AS)	FUW-IRESDsRed	TA**GAATTC**GGCCGCCTACTGAAC AGG	pIRES2DsRed -Express2
DsRed.BamHI (S)	FUW- DsRed	AT**GGATCC**CCACCATGGATAGCAC TGAG	pIRES2DsRed -Express2
DsRed (AS)	FUW-DsRedIRESRenilla (DsRed)	CTACTGGAACAGGTGGTGGCG	pIRES2DsRed -Express2
DsRed.IRES (S)	FUW-DsRedIRESRenilla (IRES)	CCACCACCTGTTCCAGTAGGCCCC TCTCCCTCCC	pIRES2DsRed -Express2
IRES.Renilla (AS)	FUW-DsRedIRESRenilla (IRES)	GTCGTACACCTTGGAAGCCATGGT TGTGGCCATATTAATCATCGTG	pGL4.70
Renilla (S)	FUW-DsRedIRESRenilla (Renilla)	ATGGCTTCCAAGGTGTACGAC	pGL4.70
Renilla.EcoRI (AS)	FUW-DsRedIRESRenilla (Renilla)	GAC**GAATTC**ATTACTGCTCGTTCT TCAG	pGL4.70

Table XII. (*Continued)*

Name	Construct (gene amplified)	Primer sequence (5'→ 3')	Template
IRES.TK (AS)	FUW-DsRedIRESTK (IRES)	TGACAGGGGTAGCTGGCCATGGTT GTGGCCATATTATCATCGTG	pIRES2DsRed -Express2
TK (S)	FUW-DsRedIRESTK (TK)	ACCATGGCCAGCTACCCCTGTCA	TK.007
TK.EcoRI (AS)	FUW-DsRedIRESTK (TK)	TGTTAA**GAATTC**ATCAGTTGGCCT CGCCCATCT	TK.007
DsRed.DTA (AS)	FUW-DsRedTK (DsRed)	CTGGAACAGGTGGTGGCGG	pIRES2DsRed -Express2
DsRed.TK (S)	FUW-DsRedTK (TK)	CGCCACCACCTGTTCCAGGCGGCC AGCTACCCCTGTCACC	TK.007
TK.BamHI (S)	FUW-TKDsRed (TK)	GA**GGATCC**CACCATGGCCAGCTA C CCCTGT	TK.007
TK.DsRed (AS)	FUW-TKDsRed (TK)	GCCTCGCCCATCTCCCGGGCGAAG	TK.007
TK.DsRed (S)	FUW-TKDsRed (DsRed)	GAGATGGGCGAGGCCAACGCGGA TAGCACTGAGAAC	pIRES2DsRed -Express2
DsRed. EcoRI (AS)	FUW-TKDsRed (DsRed)	AGAGT**GAATTC**CGCCTACTGGAA C AGGTGGTGG	pIRES2DsRed -Express2
Renilla.BamHI (S)	FUW-RenillaIRESTK (Renilla)	GTT**GGATCC**GCCACCATGGCTTCC AAG	pGL4.70
Renilla (AS)	FUW-RenillaIRESTK (Renilla)	GACTCTAGAATTACTGCTCGTTCT TCAGCAC	pGL4.70

Table XIII. Primers used for real-time PCR.

Name	Primer sequence (5'→ 3')
Actin (S)	CTGGAACGGTGAAGGTGACA
Actin (AS)	AAGGGACTTCCTGTAACAACGCA
Renilla.nt 551(S)	TCGAGTCCTGGGACGAGTGG
Renilla.nt 700 (AS)	CAGCGAACTCCTCAGGCTCC

Appendix B – optimization of transduction

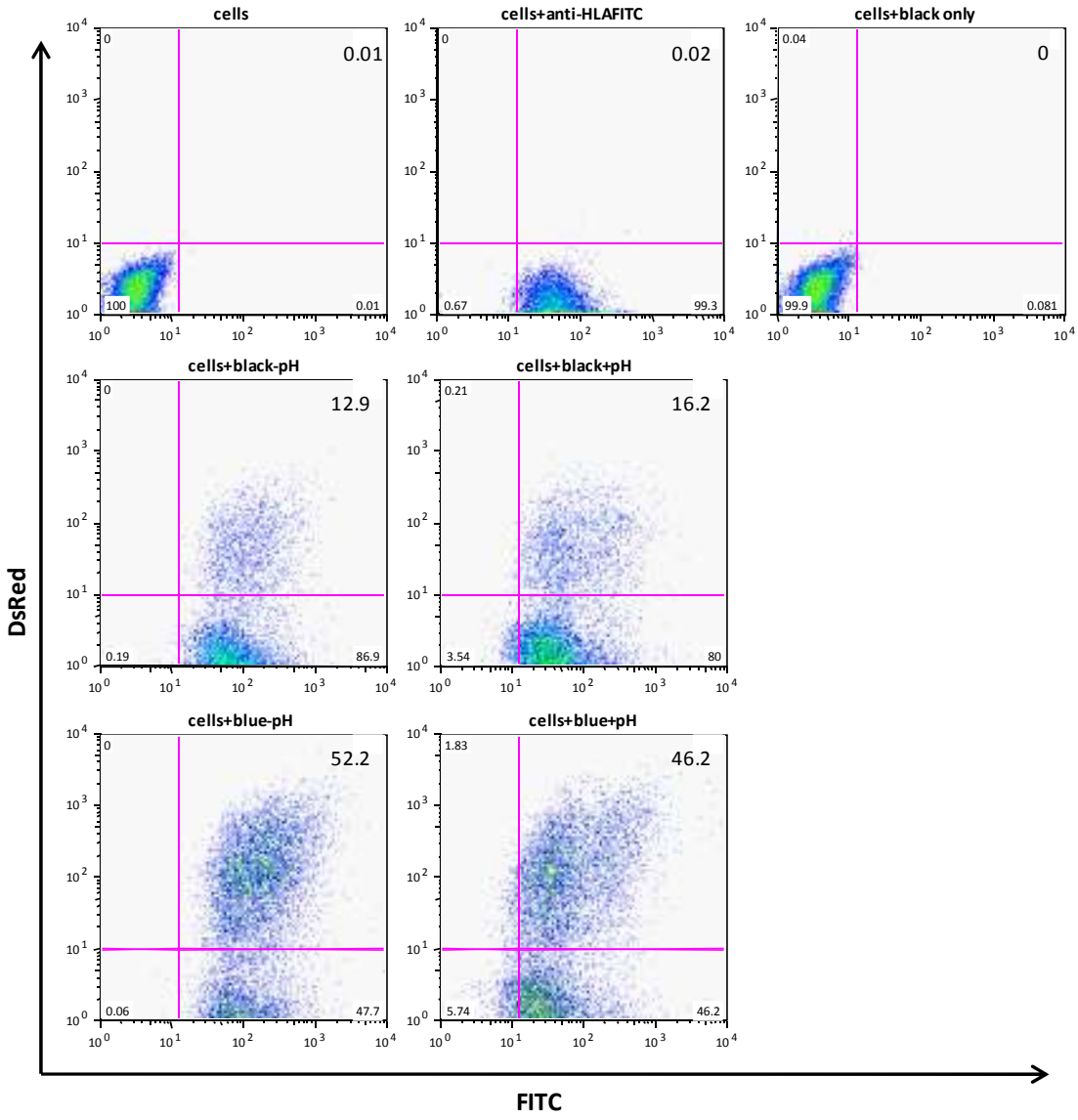

Figure 46. Optimization of *in vitro* viral transduction using Sindbis/ZZ pseudotyped lentiviral vectors expressing DsRed. Two different conditions, with different amounts of plasmids were used for viral production. (Blue: 1 µg Gag/pol; 0.32 µg Rev; 1.4 µg FuWDsRed; 0.7 µg Sindbis/ZZ); Black: 1.2 µg Gag/pol; 0.38 µg Rev; 0.38 µg FuWDsRed; 1.2 µg Sindbis/ZZ). Each virus (130 ng HIV p24) was used to transduce Jurkat cells with or without retronectin treatment to the plates (60 µg/mL) and each of this subjected or not to an acidic pH treatment. Anti-HLA-FITC (5µg/ml) was used for redirecting viral infection. Forty-eight hours after medium change, cells were analysed by flow cytometry.

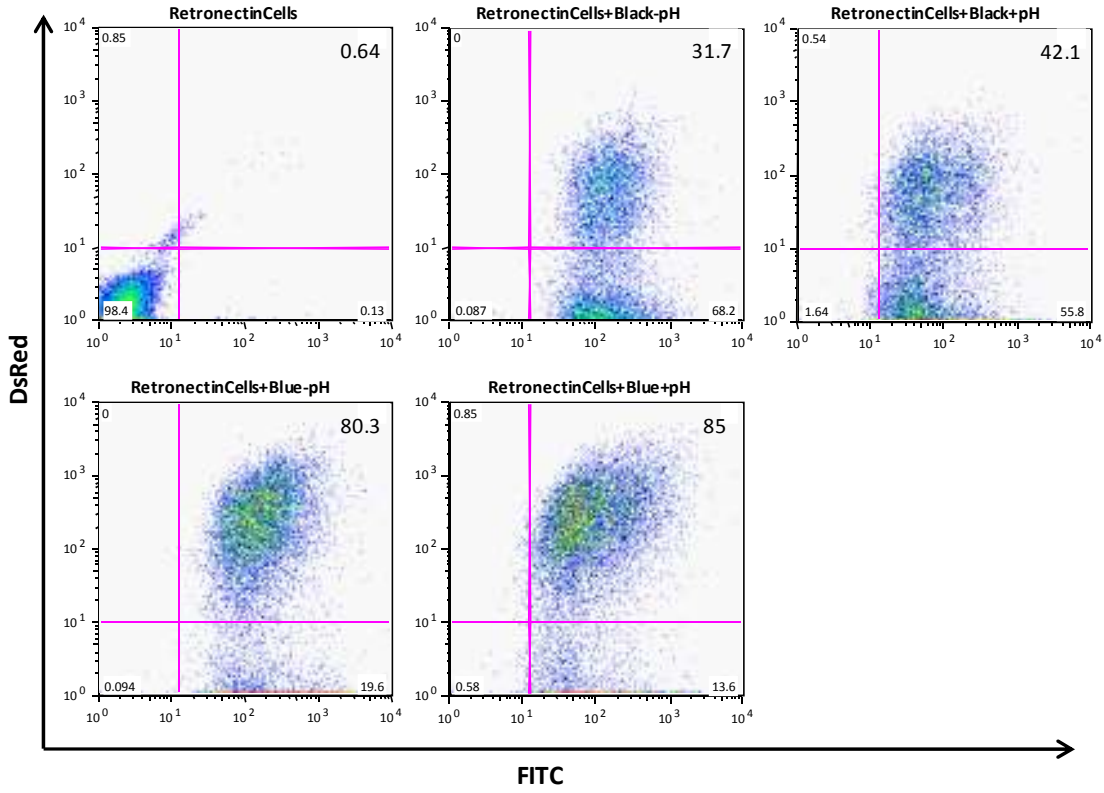

Figure 46. ***Continued.***

Appendix C – comparison of viral concentration methods

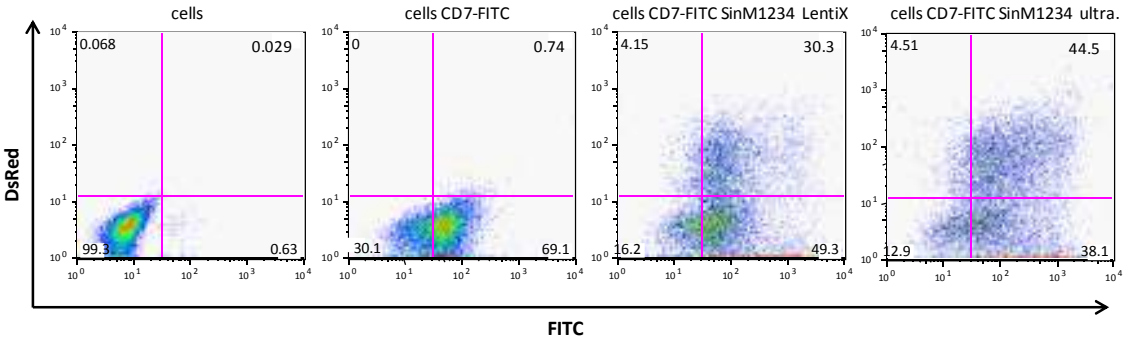

Figure 47. Comparison between transduction efficiency mediated by virus concentrated by ultracentrifugation or by Lenti-X concentrator. Sindbis/anti-FITC M1234 pseudotyped lentiviruses were produced in 293T cells and viral supernatant was divided in two parts that were concentrated either by ultracentrifugation or by the Lenti-X concentrator. Same volume of virus was used to transduce Jurkat cells in retronectin treated plates and subjected to an acidic pH treatment. Anti-CD7-FITC (5µg/ml) was used for redirecting viral infection. Forty-eight hours after medium change, cells were analysed by flow cytometry for the percentage of $DsRed^{+}$ cells.

Appendix D – comparison of the transduction efficiency between Molt-4 and Jurkat cells

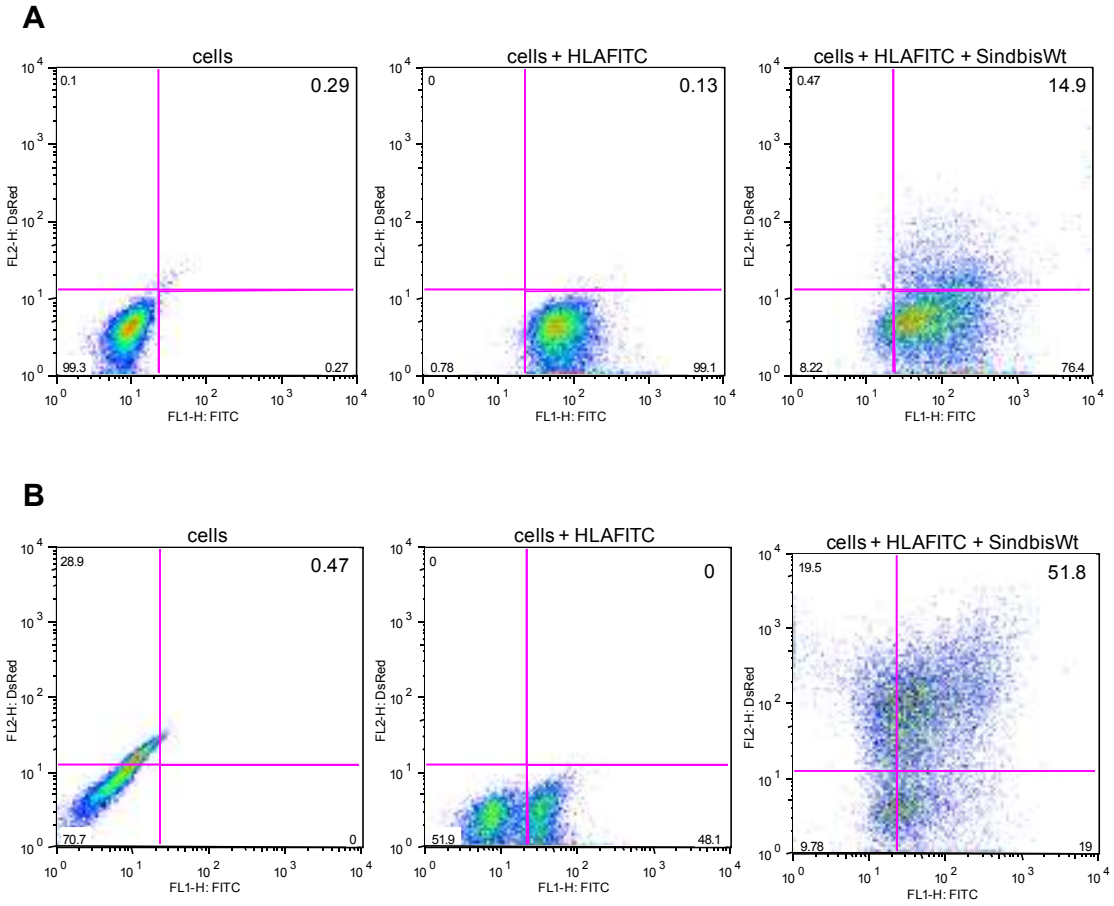

Figure 48. Comparison between transduction efficiency of Molt-4 and Jurkat mediated by Sindbis/anti-FITC Wt pseudotyped lentiviruses. Sindbis/anti-FITC Wt pseudotyped lentiviruses were produced in 293T cells and viral supernatant was concentrated by ultracentrifugation. Approximately 200 ng (HIV p24) of virus were used to transduce Molt-4 cells (A) or Jurkat cells (B) in retronectin treated plates (40 µg/mL) and subjected to an acidic pH treatment. Anti-HLA-FITC (5µg/ml) was used for redirecting viral infection. Forty-eight hours after medium change, cells were analysed by flow cytometry for the percentage of $DsRed^{+}$ cells.

Appendix E – analysis of reporter gene expression from the plasmids FUW/DsRedIRESRenilla and FUW/RenillaIRESTK, in 293T cells

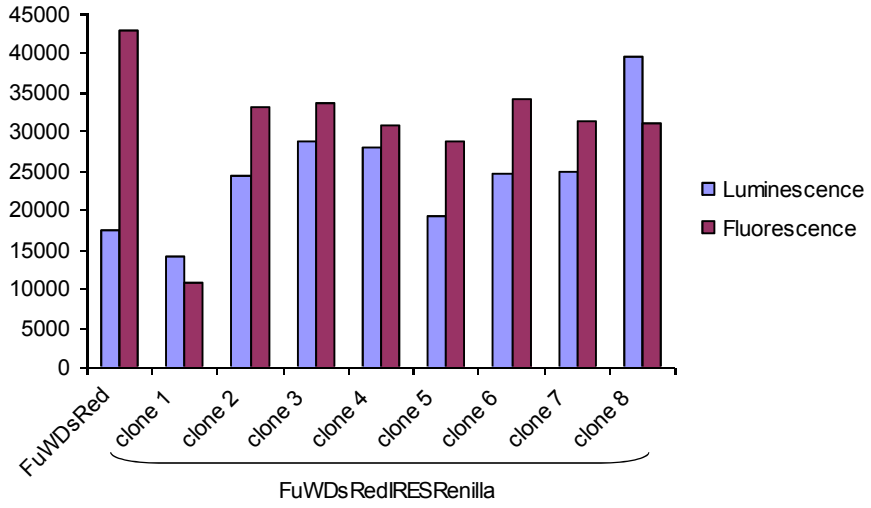

Figure 49. Analysis of the reporter gene expression of several FUW/DsRedIRESRenilla clones *in vitro* in 293T cells. Eight clones were selected for transfection of 293T cells and further analysis of the renilla luminescence and DsRed fluorescence, on the Infinite 200 device. FUW/DsRed was included as a control. Clone number eight was the one chosen for the future experiments.

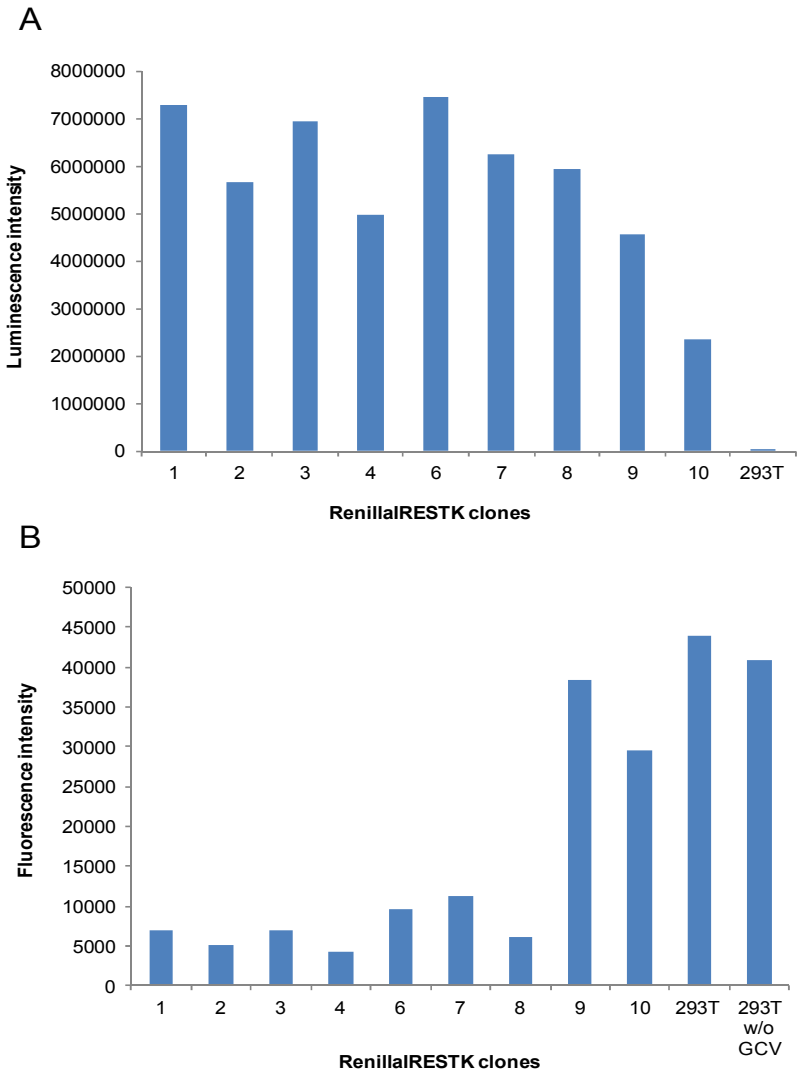

Figure 50. Analysis of the reporter gene expression of several RenillaIRESTK clones *in vitro* in 293T cells. Ten clones were selected for transfection of 293T cells and further analysis of the renilla luminescence (A) and the cell viability by AlamarBlue assay (B), on the Infinite 200 device. Non-transfected 293T cells were included as a negative control. Clone number one was the one chosen for the future experiments.

Appendix F – analysis of the sensitivity of the untransduced cell population to GCV treatment

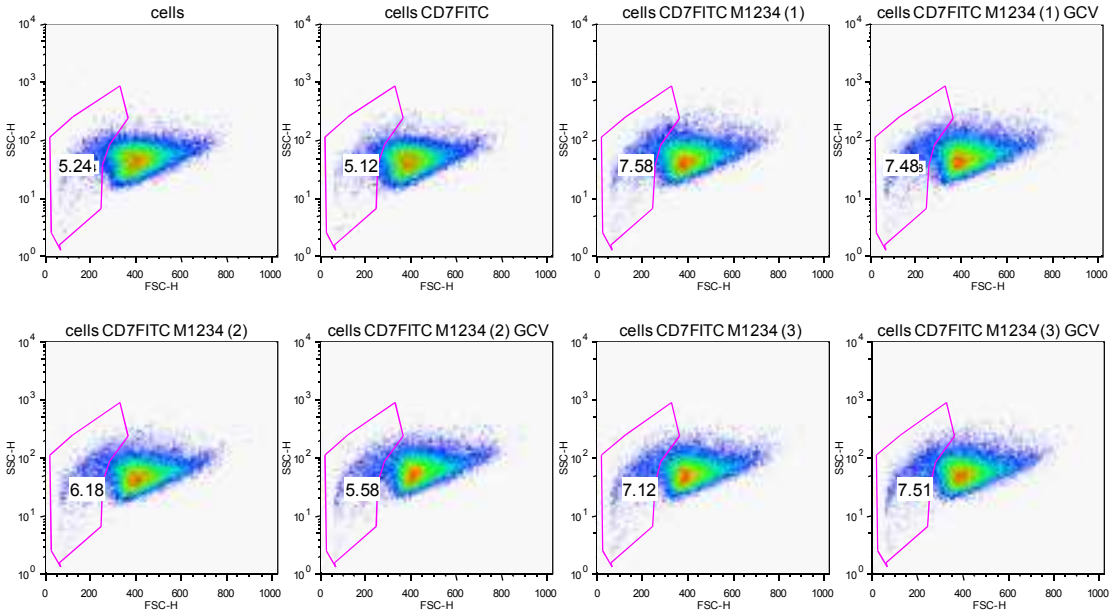

Figure 51. GCV has no effect on the untransduced cell population. Untransduced Jurkat cells corresponding to day 8 of the experiment on Figure 28 were plotted for forward and side scatter. There were basically no changes in the percentage of dead cells.